DOCTOR OF PHILOSOPHY

Identification of new functional resistance genes against P. infestans in Solanaceae species

Van Weymers, Pauline S. M.

Award date:
2016

Identification of new functional resistance genes against *P. infestans* in *Solanaceae* species

Pauline S. M. Van Weymers

MSc of Plant Biology and Environment,
University of Montpellier, France (2012)

BSc of Cellular Biology and Physiology,
University of Lille, France (2010)

Presenting for the degree of

Doctor of Philosophy

with

The Division of Plant Sciences, School of Life Sciences, University of Dundee

and

The Division of Cell and Molecular Sciences, The James Hutton Institute

August 2016

Conditions for Use and Duplication

Table of contents

List of Abbreviations

A.	*Agrobacterium*
aa	amino acid
AFLP	Amplified Fragment Length Polymorphism
ATP	Adenosine TriPhosphate
ADP	Adenosine DiPhosphate
Avr	Avirulance
Bp	Base pair(s)
BLAST	Basic Local Alignment Search Tool
BLASTn	Basic Local Alignment Search Tool of a nucleotide query against a nucleotide database
BR	Bulk Resistant
BS	Bulk Susceptible
BSA	Bulk Segregant Analysis
°C	degree Celcius
CBEL	Cellulose-Binding Elicitor Lectin
CC	Coiled Coil
CPC	Commonwealth Potato Collection
CRN	CRinkling and Necrosis
cv.	*cultivar*
DM	doubled monoploid *S. tuberosum* Group *Phureja* (clone DM1-3 516 R44)
DNA	DeoxyriboNucleic Acid
Dpi	Days post infiltration
dRenSeq	diagnostic *R* gene enrichment and Sequencing
DTT	DiThioThreitol
ETI	Effector Triggered Immunity
ETS	Effector Triggered Susceptibility
g	gram(s)
GFP	Green Fluorescent Protein
GP42	TransGlutaminase 42
GTEN	Glycerol Tris-hydrochloride EDTA NaCl buffer
H	Hour(s)

HR	Hypersensitive Response
Kb	Kilo bases
INRA	French National Institute of Agronomical Research
JHI	James Hutton Institute
L	Litre(s)
LRR-RLK	Leucine Rich Repeat, Receptor-Like Protein Kinase
LRR-RLP	Leucine Rich Repeat, Receptor-Like Protein
m	milli if prefix, meter if suffix
M	Molar
MAS	Marker-Assisted Selection
Mb	Mega base pair(s)
MCL	Markov Cluster Algorithm
min	minute(s)
MiSeq	Illumina next generation sequencing and analysis system
MM	Moneymaker
%MM	Percentage of MisMatch rate
N.	*Nicotiana*
NB-LRR	Nucleotide Binding Leucine Rich Repeat
NBS	Nuclear Binding Site
NLP	Nep1-Like Protein
n	nano
nr	nucleotide collection database
nt	nucleotides
OD_{600}	Optical Densities measured at 600nm
P.	*Phytophthora*
PAMP	Pathogen Associated Molecular Pattern
PBS(T) buffer	Buffer of 1X Phosphate Buffered Saline (with 0.1% Tween-20)
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PGSC	Potato Genome Sequencing Consortium
PITG	*Phytophthora infestans* gene annotation number
PRR	Pattern Recognition Receptor
PTI	PAMP-Triggered Immunity
PVX	Potato Virus X

PVY	Potato Virus Y
R	Resistance
RFLP	Restriction Fragment Length Polymorphism
RenSeq	*R* gene enrichment and Sequencing
RGA	Resistance Gene Analog
ROS	Reactive Oxygen Species
Rpi	Resistance to *P. infestans*
rpm	rotation per minute
s	second(s)
S.	*Solanum*
SCR	Small Cysteine-Rich
SDW	Sterile Distilled Water
SNP	Single Nucleotide Polymorphism
SSR	Simple Sequence Repeat
TGC	Tomato Genome Consortium
TIR	Toll Interleukin Receptor
TVR	Tobacco Rattle Virus
µ	micro
USDA	United States Department of Agriculture
UV	Ultraviolet
V	Volt(s)
W	Watt(s)

List of Figures

List of Tables

Acknowledgements

I first came to Dundee in March 2011, as a 1st year of Master student. I was looking for a four months internship in plant Biology, to complete my French course. A little more than 5 years later, here am I, still in Dundee and presenting my PhD thesis… One thing is for sure, it will have proven to me that little decisions can open the doors to massive opportunities, when you are lucky enough to be surrounded by the right people!

I would naturally want to start by thanking my three supervisors; Paul Birch, Ingo Hein and David Cooke; for putting their trust in me and offering me this project. I would also want to thank them for their help and support during the last four years, as I could have not done it without their guidance. A special thanks also goes to my PhD committee members, Steve Whisson and Nicola Holden, for all of their ideas and advice throughout our regular meetings.

I would like to thank the USDA for funding my project, and all our collaborators in the US, particularly John Scott and Howard Judelson, for their project inputs and friendliness. This work could have not happened without the collaboration of Véronique Lefebvre and René from INRA Avignon, France, who provided my main plant material. Same goes with Jack Vossen, Yuling Bai and teams, who gave me valuable help and support, providing me with constructs and unpublished information.

I would like to thank all the staff and colleagues from the JHI and the UoD, for helping along the way and making me feel so welcome. In alphabetical order for fairness, and

hoping I am not omitting anybody, I a particular big thank you to Alison, Audrey, Barbara, Brian, Carmen, Charlotte, Chen, Edgar, Elli, Florian, François, Fraser, Gaëtan, Gaëtan v.2, Hazel, James, Jocelyn, Katie, Laura, Lea, Lilian, Lydia, Malcom, Micha, Miles, Natalja, Patricia, Pete, Remco, Sean, Shirley, Sonia, Sophie, Stefan and Tiago. It might have been a construct, a helpful piece of advice, an extra pair of hands, a different perspective to my work or just a friendly face, you all helped me along the way and working (but also relaxing!) around you was a great pleasure.

Finally, I would like to thank my friends and family for their priceless support during the last four years. To my parents, thanks for having always encouraged me to go forward in life. It is not always easy to be so far away, but you have always been there to support me, whatever I needed and whenever I needed it, and I am truly grateful for that. (À mes parents, merci de m'avoir toujours poussée à aller de l'avant. Ce n'est pas facile d'être aussi loin, mais vous avez toujours su m'apporter votre soutien quand il le fallait et comme il le fallait. Et pour cela je ne vous remercierais jamais assez.) To Ruari, Nova, Ghillie, Frankoï and Sooty, thank you for being there. You have been so supportive through my PhD, by helping me relax and cheering me up when things were not going as smoothly as I would have liked.



Declaration

The results presented here are of investigations conducted by myself. Work other than my own is clearly identified with references to relevant researchers and/or their publications. I hereby declare that the work presented here is my own and has not been submitted in any form for any degree at this or any other university.

Pauline S. M. Van Weymers

We certify that Pauline S. M. Van Weymers has fulfilled the relevant Ordinance and Regulations of the University Court and is qualified to submit this thesis for the degree of Doctor of Philosophy.

Dr. Ingo Hein,

Cell and Molecular Sciences,

The James Hutton Institute.

Dr. David E. L. Cooke,

Cell and Molecular Sciences,

The James Hutton Institute.

Prof. Paul R. J. Birch,

College of Life Sciences,

University of Dundee

Abstract

Pests and pathogens represent a serious and continuing threat to potato and tomato production worldwide. In this thesis, I have developed a new NB-LRRs probe library accounting for the recent improved annotations of both potato and tomato (Jupe et al., 2013 and Andolfo et al., 2014). The probe library was successfully used to map a late blight resistance in the diploid potato population B3C1HP. Using bulked-segregant resistance gene enrichment and sequencing (RenSeq) analysis in this population, which segregated 1:1 for the phenotype, the resistance was mapped to the lower end of chromosome 9. Furthermore, I developed a novel diagnostic tool, dRenSeq, to screen existing germplasm collection for the presence or absence of known, already characterised disease resistance genes, to prioritise novel resistances for research and breeding. dRenSeq was applied successfully on a set of *S. okadae* accessions as a proof of concept. The tomato late blight resistance gene *Rpi-Ph3* was another focal point in this work, and the use of RenSeq was envisaged to identify *Rpi-Ph3*. However, another team published the gene (Zhang et al., 2014) and efforts were redirected towards the development of PCR markers to aid marker-assisted selection in breeding programs and to identify the cognate avirulence gene, *Avr-Ph3*. In addition, the new probe library was assessed *in silico* to evaluate if it would have enabled the identification of *Rpi-Ph3* and homologous sequences. The identification of Avr-Ph3 was established through a large effector screen in an association panel of tomato accessions, co-infiltrations with *Rpi-Ph3* in the model *Solanaceae* plant *Nicotiana benthamiana* and pathogen assays. The effector screen required the prior establishment of a robust transient expression system in tomato.

Chapter I: General Introduction

Potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*) are two important food crops from the *Solanaceae* family. Both species are prone to susceptibility towards the devastating late blight disease caused by the oomycete pathogen *Phytophthora infestans*. Resistances found in some wild *Solanaceae* species provide environmentally-benign means of restricting late blight infections in agriculture. Numerous resistance genes have already been identified in potato, whereas far fewer have been characterised in tomato. In order to make advances in controlling late blight, it is important to understand the different aspects of the plants, the pathogen and their interactions. This chapter aims to give an overview of those different aspects and to summarise the current knowledge about *P. infestans* inducible plant immune responses.

I. Plant defences against pathogens

1. Non-inducible resistances

Most plants are resistant to microbes and potential pathogens due to non-inducible defence mechanisms. Indeed, plants contain physical barriers such as cuticles (Chisholm et al., 2006) and often pre-formed antimicrobial compounds, such as phytoanticipins, that offer some protection against microbes (Gonzalez-Lamothe et al., 2009; Huckelhoven, 2007). However, some pathogens manage to penetrate these barriers or use natural openings in the plant surface, such as stomata or wounds (Huckelhoven, 2007). Nevertheless, in addition to pre-formed barriers, plants are also able to recognise pathogen attack and trigger a range of inducible defence responses.

2. Induced resistances

Plants possess an innate immune system (Jones and Dangl, 2006, Spoel and Dong, 2012), which is activated upon recognition of microbes, pathogens or cellular damage. This immune system has been well described and is divided in two levels of recognition. The Zig-Zag model by Jones and Dangl (2006) illustrates two layers of inducible defences that have been established as a consequence of plant/pathogen co-evolution. Hein et al (2009b) adapted this model to describe the specific interaction between *Phytophthora infestans* and its *Solanum* host species (Figure I.1).

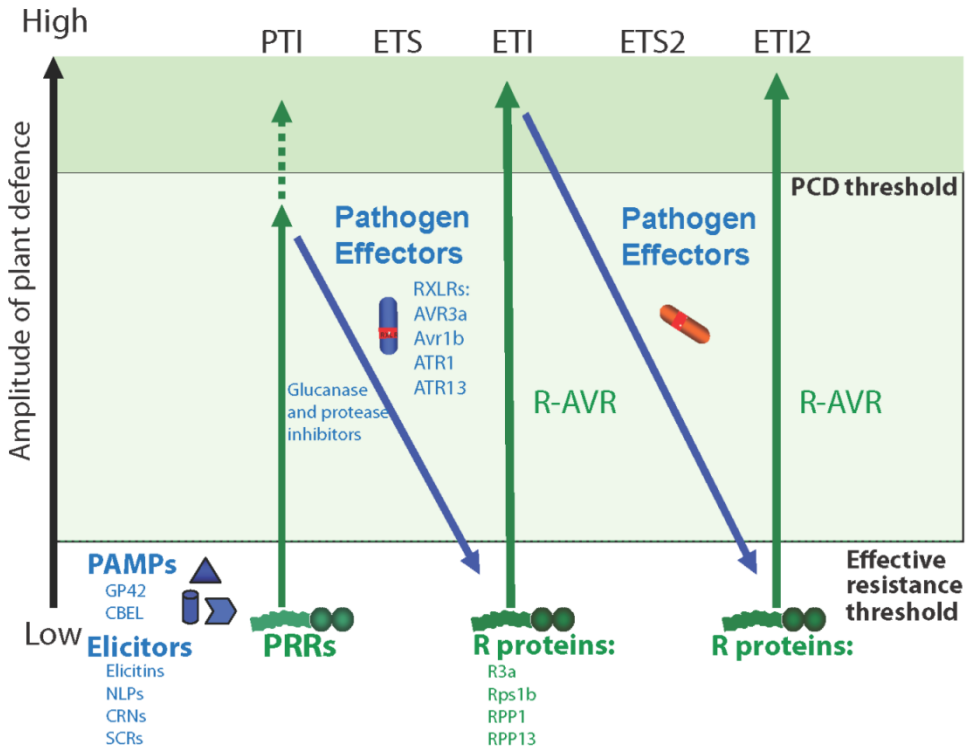

Figure I.1: The zig-zag model describing oomycete–plant interactions (From Hein et al 2009b; modified from Jones and Dangl, 2006). Shown are the characterized oomycete pathogen-associated molecular patterns (PAMPs) and other elicitors of PAMP-triggered immunity (PTI) [represented by a dotted arrow extending PTI beyond the threshold for host programmed cell death (PCD)]; examples of oomycete effectors that contribute to effector triggered susceptibility (ETS); and examples of host resistance proteins that detect oomycete effectors to trigger immunity (ETI). ETS2 and ETI2 represent a second ETS and ETI, respectively. The amplitude of defence is shown on the y axis, and the threshold for activation of host PCD is also indicated. CBEL: cellulose-binding elicitor lectin; CRN: crinkling and necrosis; NLP: Nep1-like protein; PRR: pattern recognition receptor; R: resistance; SCR: small cysteine-rich; GP42: Transglutaminase.

A. PAMP-Triggered Immunity (PTI)

The first layer of the inducible plant immune system is triggered by a broad recognition of pathogens. Indeed, it is based on the recognition of Pathogen Associated Molecular Patterns (PAMPs), which are typically highly conserved molecules essential for the pathogen survival

(Medzhitov and Janeway, 1997). The most commonly known PAMPs are flagellin from bacteria and chitin from fungi. The recognition is mediated through Pattern Recognition Receptors (PRRs), which are plant trans-membrane proteins (Zipfel, 2008). Once a pathogen has been recognised, the plant triggers a range of defence reactions which leads to PAMP-Triggered Immunity (PTI), also called basal resistance. This includes the induction of antimicrobial compounds like reactive oxygen species (ROS) and phytoalexins, or the reinforcement of cell walls through deposition of callose and lignification (Jones and Dangl, 2006; Hückelhoven, 2007). However, some adapted pathogens have evolved to evade or counteract this recognition by secreting molecules known as effectors. Nevertheless, PTI responses are utilised in agriculture by treating crops with isolated defence elicitors prior to pathogen challenge to induce resistance (Wiesel et al., 2014).

B. Effector Triggered Susceptibility (ETS)

To neutralise PTI, adapted pathogens have developed secreted molecules called effectors to promote virulence (Hein et al., 2009b; Nowicki et al., 2012; Spoel and Dong, 2012). When an effector successfully suppresses PTI, disease can occur in a process known as Effector-Triggered Susceptibility (ETS) (Jones and Dangl, 2006).

Effectors are classified within two categories, depending on their localisation in the plant. Some effectors are secreted into the plant cell (intracellular) and are thus called cytoplasmic, with some effectors having a specialised cell compartment localisation. In *P. infestans*, they are characterised by an N terminal signal peptide followed by a peptide which is involved in their trafficking inside the host cell (Birch et al., 2008, Whisson et al., 2007), and a C terminal domain involved in the manipulation of host defences (Birch et al., 2008; Bos et al., 2006). These N terminal peptides can be of two types in oomycetes, RXLR or LXLFLAK (Haas et al., 2009; Kamoun 2006). Other effectors are secreted into the plant apoplast (extracellular) and are referred to as apoplastic (Kamoun 2006). Apoplastic effectors have N terminal signal

peptides for secretion and a C terminal effector module that is often cysteine rich (Damasceno et al., 2008; Tian et al., 2007).

C. Effector Triggered Immunity (ETI)

To counteract the pathogens adaptation to PTI, plants have evolved a second layer of inducible defences through resistance (R) proteins. These proteins are able to recognise specific effector proteins, either directly according to the gene-for-gene hypothesis (Flor, 1971) or indirectly by their action on a plant target, in line with the Guard Hypothesis (van der Biezen and Jones, 1998; Dangl and Jones, 2001). Examples of both hypotheses have been identified in the past and include the flax rust effectors *AvrL567* recognised directly by the resistance protein *L5* (Ellis et al, 2007 a and b) illustrating the gene-for-gene hypothesis, or the tomato *Prf/AvrPto/Pto* indirect recognition, with *Prf* being the recognition protein (van der Biezen and Jones, 1998), illustrating the Guard Hypothesis. Furthermore, the decoy model (van der Hoorn and Kamoun, 2008) has been postulated more recently as an adaptation of the Guard Hypothesis. The Guard Hypothesis proposes that one single R protein can guard one effector target (called a hub) recognising multiple effectors or multiple effector targets (called guardees) which are in both cases indispensable for the virulence of the effector protein in the absence of the cognate R protein. The decoy model postulates that a guardee can develop into a decoy to either stop effectors from manipulating their intended target and thus functions as a dominant-negative protein, or, in the presence of functional R genes, to facilitate recognition of effectors (van der Hoorn and Kamoun, 2008).

Regardless of the mechanisms of effector recognition by *R* gene products, the resulting resistance is known as Effector Triggered Immunity (ETI). ETI often results in a hypersensitive response (HR) (Dangl et al., 1996), a form of programmed cell death (PCD). Indeed, the HRs stop biotrophic and hemi-biotrophic pathogens, as those require living plant tissue to establish disease (Dangl and Jones, 2001). The recognised pathogen effectors are referred to as avirulence (Avr) proteins, as they are no longer able to promote the virulence of the

pathogens (Hein et al., 2009; Spoel and Dong, 2012; Jones and Dangl, 2006). In contrast, unrecognised effectors are referred to as virulence proteins.

Amongst other proteins such as Bs3 which encodes a flavin monooxygenase (Römet et al, 2007), resistance genes and receptors can code for three different classes of proteins, including the Leucine Rich Repeat, Receptor-Like protein Kinase (LRR-RLK) (Afzal et al., 2008; Gish and Clark, 2011; Matsushima and Miyashita, 2012); the Nucleotide-Binding, Leucine Rich Repeat proteins (NB-LRR) (Jupe et al., 2012; McHale et al., 2006); and the Leucine Rich Repeat, Receptor-Like Protein (LRR-RLP) (Diévart and Clark, 2004; Matsushima and Miyashita, 2012). The largest family of plant *R* genes encode NB-LRR proteins (Jones and Dangl, 2006; Spoel and Dong, 2012; Meyers et al., 1999). More than 200 different NB-LRRs have been identified within the genome of the model plant *Arabidopsis thaliana*, representing more than 1% of its genome (Meyers et al., 1999). Recent advances in sequencing techniques have allowed the better annotation of NB-LRRs in species of agronomical importance, such as potato or tomato. Indeed, 755 different NB-LRRs have been annotated within the doubled monoploid *S. tuberosum* Group *Phureja* clone DM1-3 516 R44 genome (Jupe et al., 2012; 2013) and 397 NB-LRRs with the *S. lycopersicum* linage Heinz 1706 (Andolfo et al., 2013; 2014).

The NB-LRR proteins are typically composed of three main domains. The N terminal domain can be of two kinds, and subdivides the NB-LRR family into two categories of proteins. Some NB-LRRs carry a Toll Interleukin Receptor (TIR) which is a protein-protein interaction domain (Qureshi et al., 1999). Other NB-LRRs can contain a Coiled Coil (CC) domain, which is not fully conserved within the R proteins, and is highly variable (Baker et al., 1997; Lupas 1996). The central part of the NB-LRR proteins is the NB-ARC domain, which contains conserved motifs forming a functional nucleotide binding pocket (Albrecht and Takken, 2006; McHale et al., 2006; van der Biezen and Jones, 1998). This binding site is able to bind ATP, which is thought to activate the signal transduction of the R protein, whereas hydrolysis to ADP retains the

protein in an inactive state (Tameling et al., 2002). The C terminal end of NB-LRRs is made of leucine-rich repeats that contain the conserved sequence LxxLxxLxxLxLxx(N/C/T)x(x)LxxIPxx (Jones et al., 1997). This domain has been shown to be the origin of the recognition specificity of the pathogen effectors (Dodds et al., 2006; Ellis et al., 2007b; Kamoun 2006).

3. Plant/pathogen co-evolution

Plants and adapted pathogens are closely entwined in their evolution to defeat each other. This co-evolution causes a succession of ETI and ETS responses that is represented by the Zig-Zag-Zig model (Hein et al., 2009) (Figure I.1). Different mechanisms are involved in the *R* genes and effector gene co-evolution. As mentioned above, NB-LRR proteins represent the largest class of R proteins. Two thirds of the NB-LRR genes in *A. thaliana* are found in clusters, many of which contain closely related genes (Meyers et al., 2003). The same has been observed in potato and tomato (Jupe et al., 2013; Andolfo et al., 2014). This reflects the NB-LRRs evolutionary process, involving tandem duplications followed by diversification, which has been described as a birth-and-death model (Meyers et al., 1998; Bergelson et al., 2001). This close physical relationship between most of the *R* genes also facilitates genetic mechanisms, including equal and unequal crossing over (Devos et al., 2002; Leister 2004), which promotes *R* gene diversification. This goes alongside other mechanisms such as illegitimate recombination, gene conversion, point mutations, or retro-transposon activity. Whether they are fast or slow evolving, two types of *R* genes can be identified (Kuang et al., 2004). The type I *R* genes are characterised by frequent sequence exchanges between paralogs, facilitating a fast evolution. In contrast, type II *R* genes rarely undergo sequence exchanges (Friedman and Baker 2007), which imply a much slower evolution. The selection on *R* genes can either be purifying or diversifying. Purifying selection aims to maintain the R protein function using predominantly synonymous nucleotide substitutions, which do not alter the amino acid composition. At the other extreme, diversifying selection tends to create new recognition specificities through mainly non synonymous nucleotide substitutions,

which change the amino acid (aa) composition (Hein et al., 2009). The same is often observed for pathogen effectors. Effectors that are normally involved in promoting virulence but are targets for the plant immune system are predicted to be more likely to be under diversifying selection, whereas effectors implicated in the biochemical function of the pathogen are more likely to be under purifying selection (Jones and Dangl, 2006).

Regardless of the gene diversification mechanisms, plant-pathogen co-evolution is either described as an “arms race” or a “trench warfare” (Stahl et al., 1999; Bergelson et al., 2001, Hein et al., 2009). In a trench warfare scenario, a continuing adaptation (balancing selection) occurs in response to co-evolution and the diversity of pathogen effector variants is matched by the diversity of cognate host *R* genes. This scenario yields long lived *R* genes and variances and is, for example, typified by the *Hyaloperonospora parasitica* effector ATR13 and the corresponding *A. thaliana* resistance gene *RPP13* (Allen et al 2008). In a typical arms race scenario, a series of selective sweeps ensure a rapid turnover of *R* gene specificities and results in a low number of young, mainly monomorphic alleles per locus (Bergelson et al., 2001; Hein et al., 2009). A typical example for an arms race is depicted by the *Rps2* resistance gene from *A. thaliana* that governs resistance to strains of the bacterial pathogen, *Pseudomonas syringae* pv. tomato which express the cognate *avrRpt2* gene (Ding et al 2007).

4. Microbiota and disease suppression

A new aspect of plant resistance is growing within the phytopathology community. Indeed, the microbiota surrounding plant roots is believed to confer protection to those plants (Bulgarelli et al., 2013). The concept of disease suppressive soil has been developed (Kinkel et al., 2011). It describes soils in which little or no disease develops, despite conditions which should theoretically be in favour of the disease. This phenomenon can occur both naturally with the property of the soil, or after induction by a monoculture of the same species for a number of consecutive years (Berendsen et al., 2012). In the case of the natural disease suppression, the effect seems to occur for any plants. However, the induced disease

suppression shows a specificity of host-pathogen combination, which implies a primary role for the microbiota in controlling the disease (Berendsen et al., 2012). Other observations support the critical role of the microbiota in disease suppressiveness. A study showed that pasteurised suppressive soil inoculated with a given pathogen, systematically led to the reestablishment of the corresponding host disease (Mendes et al., 2011). Another study revealed that, in the case of host plants protected through disease suppressive soil against soil-borne pathogens, no particular resistance genes against those pathogens were observed (Kinkel et al., 2011). Overall, this new aspect of plant resistance is still quite obscure and requires further investigation, but promises new exciting discoveries in the coming years.

II. *Phytophthora infestans*: the plant destroyer

1. Origin and history of *Phytophthora infestans*

A. The genus *Phytophthora*

Phytophthora comes from the Greek language and translates to “plant destroyer”. Indeed, *Phytophthora* species are the most devastating pathogens on dicotyledonous plants. Their host range is diverse, including diverse genera of trees, *Solanaceae* and *Asteraceae* (Erwin and Ribeiro, 1996). *Phytophthoras* are part of the oomycete class, which is a group of fungus-like eukaryotic microorganisms (Rossman and Palm, 2006; Erwin and Ribeiro, 1996). For a long time, oomycetes have been considered fungi, but marked differences in their sexual reproduction and study of their cell wall composition (Table I.1) led to a revision of their classification (Rossman and Palm, 2006; Erwin and Ribeiro, 1996). Oomycetes are actually closer to algae than fungus, sharing attributes such as diploid vegetative cells and cellulose cell walls (Fry and Goodwin, 1997a and b).

Table I.1: Major distinctions between the oomycetes and the true fungi (Chytridiomycota, Glomeromycota, Zygomycota, Ascomycota, Basidiomycota). *From Rossman and Palm, 2006.*

Character	Oomycete	True Fungi
Sexual reproduction	Heterogametangia. Fertilisation of oospheres by nuclei from antheridia forming oospores.	Oospores not produced; sexual reproduction results in zygospores, ascospores or basidiospores
Nuclear state of vegetative mycelium	Diploid	Haploid or dikaryotic
Cell wall composition	B-glucans, cellulose	Chitin. Cellulose rarely present
Type of flagella on zoospores, if produced	Heterokont, of two types, one whiplash, directed posteriorly, the other fibrous, ciliated, directed anteriorly	If flagellum produced, usually of only one type: posterior, whiplash
Mitochondria	With tubular cristae	With flattened cristae

B. *Phytophthora infestans*, from unknown pathogen to crop threat

The geographical origin of *P. infestans* is slightly controversial. For a long time, it was thought that the potato late blight pathogen originated from Central Mexico (Fry and Goodwin, 1997a and 1997b). Conversely, several studies suggested a South American origin (Gomez-Alpizar et al., 2007, Grünwald and Flier 2005). However, a Mexican origin has recently been supported (Goss et al 2014). *P. infestans* caused the first recorded late blight outbreak in the United States in 1843 (Fry, 2008; Nowicki et al., 2012). Infected tubers were sent to Belgian farmers, which allowed *P. infestans* to cross the Atlantic Ocean in 1845 (Nowicki et al., 2012). The impacts of this disease were particularly dramatic in Ireland. Potato was then the main staple food in Ireland and *P. infestans* caused the infamous Irish potato famine in 1845. As a consequence, more than one million people died of starvation and another million people were forced to emigrate to other European countries or the USA (Fry and Goodwin, 1997 a; Nowicki et al., 2012). Until the 1980s, only the A1 mating type was present outside of Mexico (Fry and Goodwin, 1997 a and b; Fry, 2008; Foolad et al., 2008; Nowicki et al., 2012).

However, the first reported European A2 mating type of *P. infestans* was discovered in Switzerland in 1984 (Goodwin and Drenth 1997). Indeed, a second migration of *P. infestans* occurred in 1976, when Mexican potatoes were sent to Europe after a drought (Fry and Goodwin, 1997 a and b; Goodwin and Drenth 1997). This second migration introduced the mating type A2 outside Mexico, allowing sexual reproduction of *P. infestans*. This is predicted to have resulted in the emergence and the development of more aggressive isolates (Fry and Goodwin, 1997 a and b; Fry, 2008; Foolad et al., 2008; Nowicki et al., 2012). In 2004, a new genotype, *13_A2*, appeared in Europe and reached a peak in its epidemic in Great Britain in 2008, where it represented 79% of all UK fields isolates (Figure I.2) (Fry et al, 2009; Cooke et al., 2012). This particular genotype contains some of the most aggressive isolates known, capable of overcoming previously durable resistances introgressed in many potato varieties, such as the number one organic cultivar (*cv.*) Lady Balfour (Fry et al, 2009; Cooke et al., 2012). From 2004 onwards, another genotype called *6_A1* emerged in UK fields, displacing *13_A2* as the most abundant genotype in 2011 (Cooke et al., 2012).

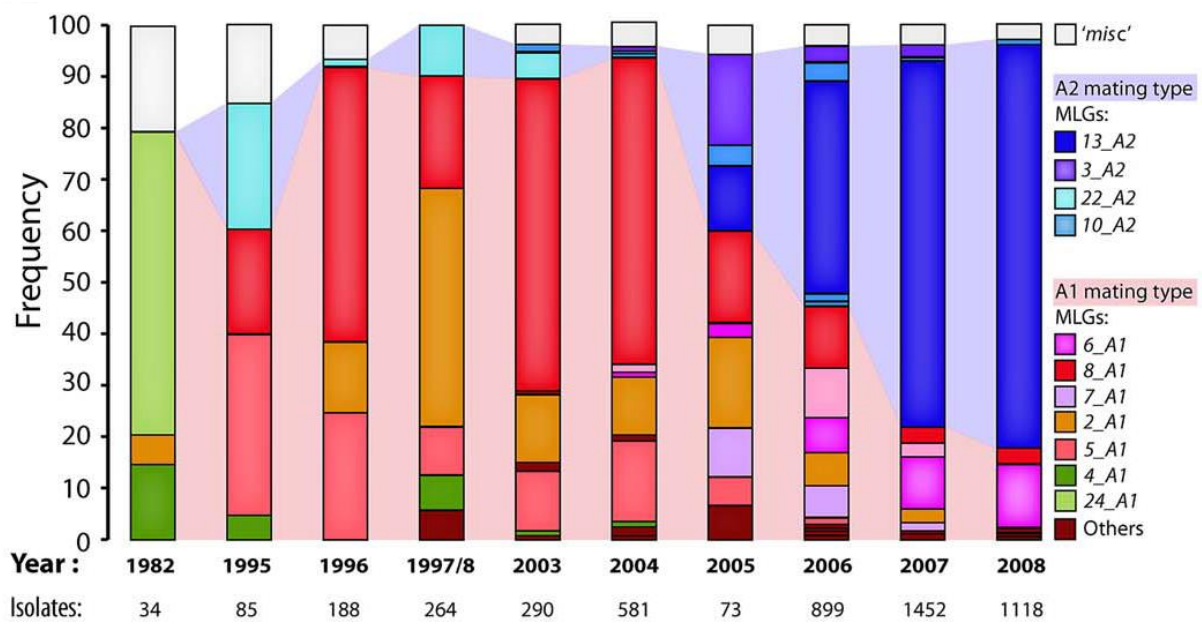

Figure I.2: *P. infestans* population displacement in Great Britain by the *13_A2* genotype. This graph shows the frequency of multilocus genotypes (MLGs) over the course of 11 years from more than 4000 infected potatoes. The number of isolates fingerprinted each year and dominant MLGs of each mating type are indicated. Isolates of MLGs that occurred at a very low frequency in a single year are grouped under the category termed 'misc'. The shading between the bars indicates the proportion of A1 and A2 mating type isolates. *From Cooke et al., 2012.*

C. *Phytophthora infestans* on the molecular level

Due to the huge economic impact of *P. infestans* on potato and tomato crops (see II.3.A.), there is now a significant effort to provide more durable late blight resistant cultivars (Vleeshouwers et al., 2011; Nowicki et al., 2012). One way to reach this goal is to better understand the pathogen and its diversity. The *P. infestans* isolate *T30-4* was sequenced in 2009 (Haas et al., 2009). Its 240 Mb genome revealed 74 % of repetitive DNA and a high level of transposons (Haas et al., 2009), which explains its high plasticity and rapid evolution to overcome resistances. The *P. infestans* genome has been described as "two speed", as it contains both gene dense and gene-sparse but repeat-rich regions, with predicted differences in evolutionary rate (Haas et al., 2009).

P. infestans secretes both apoplastic and cytoplasmic effectors and many of these are found in the gene-sparse regions (Haas et al., 2009). Intracellular effectors are translocated into the cytoplasm of host cells and can target various processes and organelles to promote virulence. Two known protein families are represented within the 753 putative cytoplasmic effectors encoded by *P. infestans* (Haas et al., 2009). One family contains the RXLR proteins, which are characterised by conserved N-terminal RXLR and often EER motifs. This dual motif has shown to be essential for the translocation of Avr3a into the plant cell (Whisson et al., 2007; Birch et al., 2008; Bozkurt et al., 2012). RXLR effectors are the most abundant family of cytoplasmic effectors in *P. infestans* with 563 putative genes (Haas et al., 2009). The second family comprises Crinkler or CRN (for Crinkling and Necrosis) proteins. These effectors are characterised by a conserved N-terminal LXLFLAK motif and approximately 196 *CRN* genes have been predicted in T30-4 (Haas et al., 2009). So far, all known and functionally characterised *P. infestans* avirulence proteins harbour the RXLR-EER motifs (Hein et al., 2009b). Two other genomes of *P. infestans* have recently been sequenced and encompass the isolates 06_3928A (genotype *13_A2)* and NL07434 (a Dutch isolate collected in 2007) (Cooke et al., 2012). Potato plants were infected separately with those two isolates and the reference T30-4, to carry out a gene expression analysis during potato infection (Cooke et al., 2012). The comparison of the induced RXLR genes during potato infection identified a set of 45 that were consistently induced in the three *P. infestans* strains (Figure I.3) (Cooke et al., 2012), representing a potential minimal set that is essential for infection. This number has since increased with the use of a more sensitive array design from Agilent. This core set of effector is considered to contain effectors important in the establishment of infection, due to its conservation within isolates. A strategy for introducing durable resistance is to identify *R* genes that specifically target effectors and known variants belonging to this core set of effectors (Birch et al 2008).

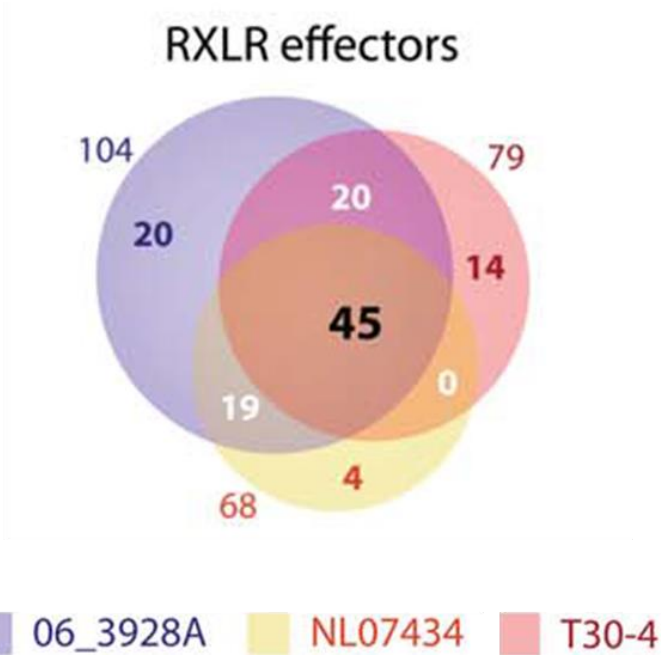

Figure I.3: Gene expression polymorphisms between *P. infestans* isolates 06_3928A, T30-4 and NL07434. The Venn diagram illustrates the number of RXLR effector genes that are induced during potato infection in *P. infestans*. Only a relatively small subset of genes is consistently induced in the three strains. *From Cooke et al., 2012.*

2. Life cycle of *P. infestans*

As *P. infestans* is a hemibiotrophic pathogen, its infection is characterised by two specific stages (Fry and Goodwin, 1997a) (Figure I.4). The first part of the lifecycle is biotrophic and characterised by the development of haustoria from the mycelium. These haustoria protrudes through the cell wall into living host cells without penetrating directly into the cytoplasm (Fry and Goodwin, 1997a and b; Fry, 2008; Nowicki et al., 2012). The second step is necrotrophic, and the pathogen causes host cells to die prior to initiating sporulation (Fry and Goodwin, 1997a and b; Fry, 2008; Nowicki et al., 2012). The late blight pathogen is able to complete its life cycle either asexually or sexually, if both mating types (A1 and A2) are present (Fry and Goodwin, 1997 a and b; Fry, 2008; Nowicki et al., 2012). Asexual reproduction requires indeterminate structures called sporangiophores, which produce and aid the passive dispersal of sporangia through wind or rain. Sporangia can then either

germinate directly on hosts, or release flagellated asexual spores called zoospores. Sexual reproduction can be initiated when two different mating types interact with each other, and form sexual spores called oospores. Unlike zoospores, which are the main disease causing agents during the season, oospores are persistent and can serve as survival structures in the ground for several years (Figure I.4). Overall, asexual reproduction allows *P. infestans* a rapid growth of its population, whereas its sexual reproduction represents a great source of variation and survival (Fry, 2008). The major agronomical problem of *P. infestans* infection is that the early biotrophic stage has no visible symptoms. Thus, farmers are only aware of the disease at the beginning of the necrotrophic phase, when the pathogen can release up to 300,000 sporangia per lesion. At this stage, late blight can destroy a field within ten days (Figure I.5) (Fry and Goodwin, 1997 a and b; Birch and Whisson, 2001; Fry, 2008; Nowicki et al., 2012). Moreover, *P. infestans* is able to infect foliage, stem, fruit and tubers, even in storage. Chemicals are most effective in preventing infection, rather than treating disease (Fry 2008), and overall are not environmentally friendly. Moreover, the European Union is limiting the use of chemicals, with for example its directive on a more sustainable use of pesticides (2009/128/EC). This shows the importance of finding resistances to breed into commercial cultivars, to act ahead of the disease in a more environmentally-friendly way.

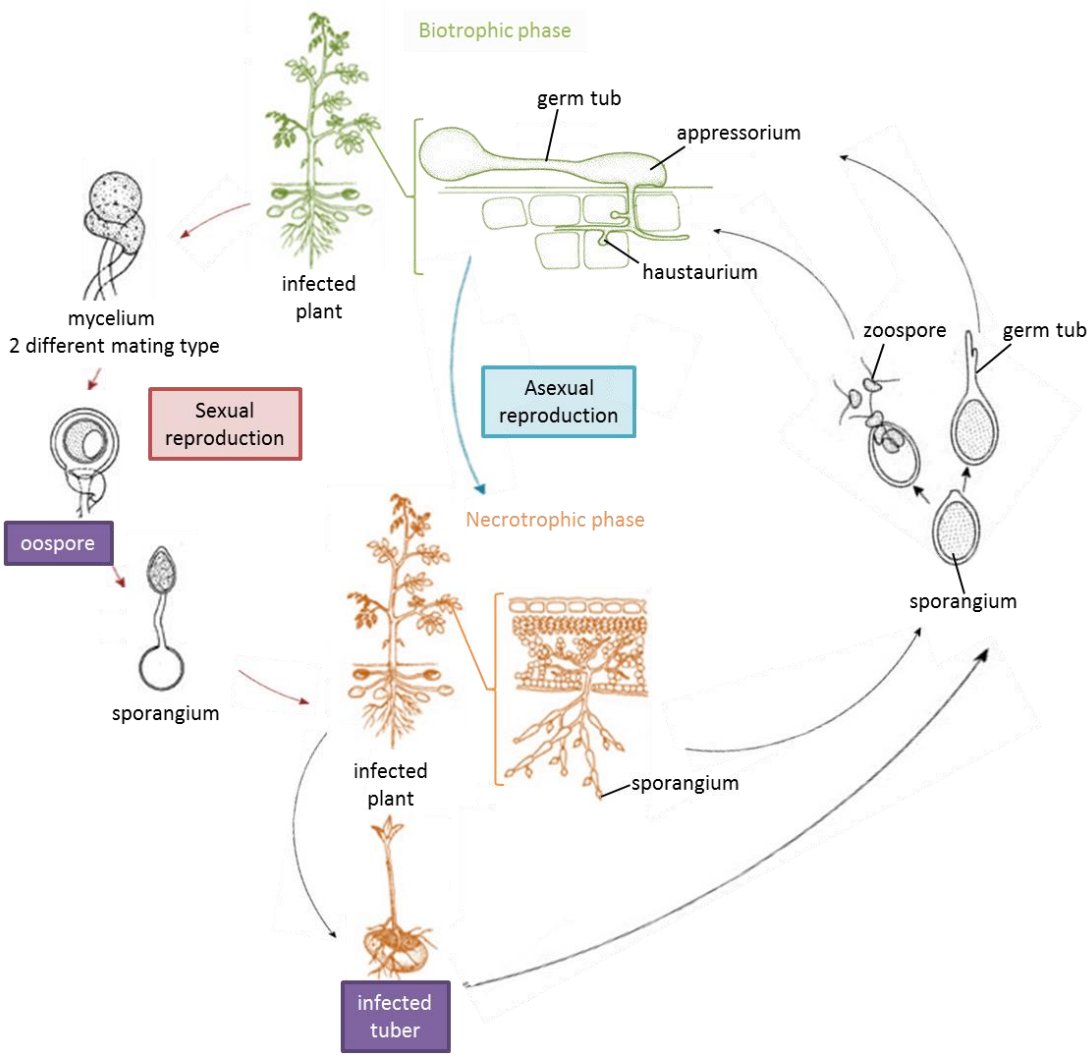

Figure I.4: Life cycle of *Phytophthora infestans*. The hemibiotrophic *P. infestans* infection is characterised by two phases. The biotrophic phase (in green) is characterised by the development of haustoria from mycelium, which prude through the cell wall into living host cells without penetrating directly into the cytoplasm. The second phase is necrotrophic (in orange), and the pathogen causes host cells to die, prior to initiating sporulation. The late blight pathogen is able to complete its lifecycle either asexually (in blue) or sexually (in red). The asexual reproduction requires indeterminate structures called sporangiophores, which produce and aid the passive dispersion of sporangia through wind or rain. Sporangia can either germinate directly on hosts or release flagellated asexual spores, called zoospores. Sexual reproduction requires the interaction of two different mating types (A1 and A2), and results in the formation of sexual spores called oospores. Zoospores are the main disease causing

agents during the season, whereas oospores are persistent and can serve as survival structures (in purple) for several years. Infected tubers are also allowing the pathogen to survive from one season to another. Adapted *from Plant Diseases: Their Biology and Social Impact, by Gail L. Schumann, 1991.*

3. Resistance against *Phytophthora infestans*

A. Importance of late blight control

P. infestans is one of the most devastating diseases of potato and tomato. Late blight can, for example, destroy up to 16% of the global potato yield each year, representing annually £55 million losses for the UK potato industry alone (Haverkort et al., 2009). This represents costs of up to 5.2 billion euros (around £ 4.4 billion) worldwide, including crop losses and the use of chemicals to contain the disease (Birch and Whisson, 2001; Whisson et al., 2001; Haverkort et al., 2009). Looking only at the crop losses on potato and tomato productions, late blight represents a loss of 6.7 billion US dollars (around £ 4.4 billion) (Fry 2008, Haas et al., 2009; Haverkort et al., 2008). Those numbers, despite being a few years old, give a good overview of the economic impact of late blight. The most common way to protect crops against *P. infestans* is the use of fungicides. Indeed; Metalaxyl combined with Carbamate, or Cymoxanil combined with Mancozeb, are the most commonly applied fungicides (Mukerji, 2004). However, since the 1980s, resistance to Metalaxyl was identified first in European and Middle East isolates of *P. infestans* (Cooke, 1981; Davidse et al., 1981; Cohen and Reuveni, 1983). Resistant isolates have also been found in North America in the 1990s and in Asia since the beginning of the 2000s (Deahl et al., 1993, Miller et al., 1997; Deahl et al., 2002). As mentioned before, in the early biotrophic phase symptoms are difficult to spot in the field. When disease symptoms are apparent, it is often too late to treat with fungicides, as the necrotrophic phase has already started. At that point, the field can be destroyed within 10 days (Figure I.5). Furthermore, with all the environmental concerns about spraying large quantities of chemicals in the fields, but also the increasing pressure from the European

Union on the use of more sustainable pesticides (directive 2009/128/EC), it is becoming important to find alternative ways to contain and even prevent late blight disease.

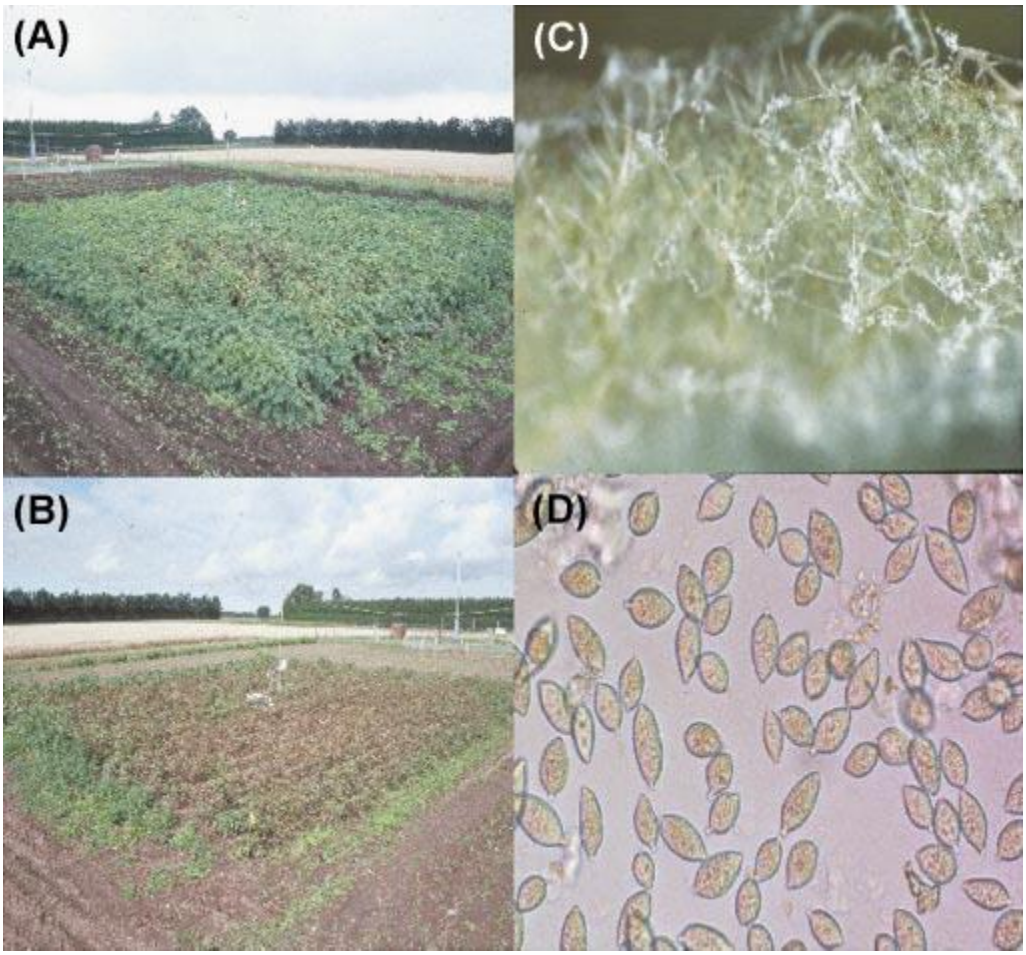

Figure I.5: Natural infection by *P. infestans* of a plot containing susceptible potato *cv.* Bintje (A and B). Early symptoms of infection can be seen in the centre of the plot (A), followed by massive devastation of the plant foliage 10 days later (B). Aerial hyphae growing from the underside of a susceptible leaf (C) develop sporangiophores which produce sporangia (D) in the later stages of infection. *From Birch and Whisson, 2001*.

B. Molecular understanding of *P. infestans* resistances

A more environmentally-benign approach to control late blight is based on deploying major *R* genes that recognise essential effectors (Birch et al 2008). The main source for new resistances is found in wild *Solanaceae* species, which have naturally co-evolved with *P. infestans*. However, the rapid evolution of *P. infestans* effectors can leads to a quick 'boom

and bust' cycle for many Resistance to *P. infestans* (*Rpi)* genes, as pathogen variations that can overcome these resistances emerge (Vleeshouwers et al., 2011). Introgressing several resistant genes into a single cultivar (*R* gene pyramiding) is thought to be a way to increase the resistance durability (Fry, 2008; Nowicki et al., 2012).

So far in the literature, all *P. infestans* avirulence genes identified are RXLR containing effectors (Armstrong et al., 2005; Lokossou et al., 2009; Oh et al., 2009; van Poppel et al., 2008; Tyler et al., 2006; Vleeshouwers et al., 2008). This motif was first thought to be a universal target for *P. infestans* recognition (Birch et al., 2008), but so far no evidence has emerged that it is targeted as a PAMP. However, this conserved motif can be used as a tool to identify potential resistances. Indeed, with the availability of three *P. infestans* genomes, comprehensive lists of predicted RXLR effectors have been defined (Haas et al., 2009; Cooke et al., 2012). These putative effectors can then be further analysed for expression during infection, but can also be screened for recognition in wild *Solanaceae* species by transient expression to identify novel *R* genes (Vleeshouwers et al., 2008 and 2011; Vleeshouwers and Oliver; 2014). Indeed, if an effector is triggering a genotype specific HR in plants, it is likely that the corresponding plants carry a cognate *R* gene (Birch et al., 2008). This method, called effectoromics (Vleeshouwers et al., 2008 and 2011; Vleeshouwers and Oliver; 2014), allowed the identification of *Rpi-sto1* from *S. stoloniferum,* for example (Vleeshouwers et al., 2008). It also demonstrated that several *R* genes like *Rpi-sto1*, *Rpi-pto1* and *Rpi-blb1* from different species –here *S. stoloniferum*, *S. papita* and *S. bulbocastanum* respectively - can recognise the same effector (Vleeshouwers et al., 2008). Thus, effector screening allows the determination of a given resistance specificity, and is a valuable tool in disease resistance breeding (Vleeshouwers and Oliver; 2014). Indeed, if considering *R* gene pyramiding for example, it wouldn't make sense to combine *Rpi* genes with the same specificity. However, knowing that the three genes confer the same resistance specificity, the breeder could choose the resistant species easier to cross with his cultivar, depending on their ploidies and

potential incompatibilities. Despite being a promising way of controlling *P. infestans* in the longer run, *Rpi* pyramiding has its limitations. Indeed, cultivars containing several *R* genes have already been infected in the field within several years after their release. A good example is the Scottish *cv.* Pentland Dell, which contains *R1*, *R2* and *R3a* from the wild potato species *S. demissum*, and which was infected six years after being released (Hein et al., 2009b). This was most likely due to a high pressure of selection on the pathogen resulting from monoculture, which caused a rapid adaptation of *P. infestans* (Fry 2008, McDonald and Linde 2002). This example shows shows the limitations of *R* gene pyramiding to control *P. infestans*, without an appropriate knowledge of the molecular mechanisms behind the resistance diploid. Indeed, a better understanding of the effector(s) recognised by a resistance gene, with the knowledge of its diversity and importance in the infection, will help establishing a more integrated and efficient way of farming.

In addition to ruling out *Rpi* genes with identical specificity, effectoromics also enables the search for more durable resistances. As mentioned above, core effectors represent potentially the pathogen's Achilles heel. Identifying universally expressed and essential effectors and studying their naturally occurring diversity can inform breeders of the best targets for resistance (Birch et al 2008; Vleeshouwers et al 2011). One such example is *Avr3a*, which has been shown to be universally expressed (Cooke et al 2012), essential for virulence (Bos et al 2010) and conserved in *different P. infestans* populations (Armstrong et al 2005; Cardenas et al 2011, Seman et al unpublished). Efforts are underway to identify and engineer *Rpi* genes that recognise all known variants of such core-effectors and it is thought that deployment of such genes in combination could lead to durable resistance. To remain with the Avr3a example, there are two forms of the effector prevalent in current *P. infestans* populations (Armstrong et al., 2005; Cardenas et al 2011). AVR3aKI is the avirulent form of the effector, and is recognised by R3a. AVR3aEM is the virulent form of this effector, avoiding

recognition by R3a. However, *R3a* resistance gene has been engineered to recognise AVR3aEM (Chapman and Stevens et al., 2014), increasing the resistance spectrum of *R3a*.

III. *Solanum* under the threat of *Phytophthora infestans*

1. *Solanum tuberosum* or potato

A. Origin and history of potato

Solanum tuberosum (*S. tuberosum*), commonly called potato, is an angiosperm eudicot plant and part of the *Solanaceae* family, belonging to the genus *Solanum*. It is a tuber-bearing *Solanum*, belonging to the section *Petota*. The number of chromosomes (ploidy) in the different potato species is variable, ranging from diploid (2n=2x=24) to hexaploid (2n=6x=72), including triploid, tetraploid and pentaploid species (Spooner and Salas, 2006). Classification of the different species is challenging, due to the great ability *Solanum* species have to hybridise, but also with the emergence of new technologies to study specification on a genomic level (Hawkes 1990; Jacobs et al., 2008, Spooner and Hijmans 2001). Hawkes (1990) counted 227 tuber-bearing species (included 7 cultivated) and 9 non-tuber-bearing in his classification of the section *Petota*. Conversely, Spooner and Hijmans (2001) recognised 203 tuber-bearing species (included 7 cultivated) within the *Petota*. Spooner and Salas (2006) identified 189 tuber-bearing species (included 1 cultivated) within the *Petota* section and more recently, Rodrigruez et al., (2010) suggested 100 different species. Even now, there are still debates whether some species should be considered as one single species or not, including the example of the closely related wild species *S. venturii* and *S. okadae* (Jacobs 2008, Jacobs et al., 2008; Pel et al., 2009).

Wild potato species originate most likely from South America. The commercial potato is derived from the wild species *S. tuberosum*, originated from the Andes (South America), where it was originally cultivated 8,000 years ago. The tetraploid groups of *S. tuberosum* are thought to be derived from hybridisations of closely related species, including the *S.*

brevicaule group (*S. bukasovii*, *S. candolleanum*), as well as *S. avilesii*, *S. berthaultii*, *S. infundibuliforme*, *S. oplocense*, *S. sparsipilum*, *S. sucrense* and *S. verrucosum* (Rodriguez et al 2010). *S. tuberosum* was first introduced in Europe in the 16th century. Indeed, in 1532, Spain was the first European country to grow potatoes, followed in 1588 and 1593 by England (Hawkes 1990). Cultivated for its edible tubers, potato became the third most consumed food crop worldwide, after wheat and rice (Vleeshouwers et al., 2011). In 2005, the estimated world potato cultivation was around 20 million hectares with a total world production of 300 million tons (Haverkort et al., 2009). Nowadays, these numbers are still increasing with a world production of 324 million tons in 2012 (FAOSTAT), showing the economic importance of potato as a crop. As the most devastating potato disease is caused by *P. infestans* and because of these economic stakes, important resources have been developed to improve resistances in potato cultivars. A high density molecular linkage map of potato has been available since 1992 (Tanksley et al., 1992). However, the recent sequencing of the doubled monoploid *S. tuberosum* Group *Phureja* (clone DM1-3 516 R44) genome provides an unprecedented insight into this crop (The Potato Genome Sequencing Consortium, 2011). The 844 Mb genome has been analysed and 39,000 genes predicted across the 12 chromosomes (The Potato Genome Sequencing Consortium, 2011).

The Commonwealth Potato Collection (CPC) is a collection of wild potato accessions collected from several expeditions to Mexico and South America since the 1930s. The CPC comprises more than 1800 potato accessions, representing more than 80 species. This collection is a way to access the natural diversity, to find potential new and more durable *Rpi* genes. It is also a source of allelic variations for genes involved in more complex traits such as yield, water-use efficiency, heat/draught tolerance and nutritional qualities.

B. Resistance gene to *Phytophthora infestans* in potato

The greatest diversity of wild resistant potatoes is found in Central Mexico, including the Toluca Valley (Vleeshouwers et al., 2011), where wild *Solanum* species and *P. infestans* are

co-evolving (Fry and Goodwin, 1997 a and b, Fry, 2008). The first wild potato species studied and exploited for its resistance to *P. infestans* was *S. demissum*. Indeed, at least 11 race-specific *P. infestans* resistance genes (*R1* to *R11*) have been reported and introgressed into cultivated potatoes from this species (Van der Vossen et al., 2003; Vleeshouwers et al., 2011; Nowicki et al., 2012) with eight of them: *R3*(*a* and *b*), *R5*, *R6*, *R7, R9*, *R10* and *R11* being closely related on Chromosome 11 (El Kharbotly et al., 1994; Huang et al., 2004 and 2005). Unfortunately, *P. infestans* isolates overcoming these resistances have been found already. A number of other wild species such as *S. bulbocastanum* have been studied for their resistance. At least three distinct resistance genes have been identified in this potato species, including *Rpi-blb1* (or *RB*) on chromosome 8, *Rpi-blb2* on chromosome 6 and *Rpi-blb3* on chromosome 4 (Song et al., 2003; Van der Vossen et al., 2003; Van der Vossen et al., 2005; Park et al., 2005; Lokossou et al., 2009). However, this *S. bulbocastanum* is not directly crossable with the cultivated *S. tuberosum*, which makes breeding difficult for these genes. Indeed, the introgression of *Rpi-blb2* via bridge crosses into the potato *cv.* Bionica and Toluca took more than 46 years to complete (Haverkort et al 2009). Allele mining of the broad spectrum *Rpi-blb1* gene solved this problem, with the discovery of orthologous genes in the crossable species *S. stoloniferum* (*Rpi-sto1*), *S. papita* (*Rpi-pta1* and *Rpi-pta2*) and *S. polytrichon* (*Rpi-plt1*) (Wang et al., 2008). Similarly, *Rpi-blb3* is closely related to *Rpi-abpt*, *R2*, *R2-like* and seven other *R* genes (Vleeshouwers et al., 2011), all of which are members of the same gene cluster on chromosome 4 (Park et al., 2005; Lokossou et al., 2009; Champouret PhD thesis, 2010). Functional *Rpi-blb2* homologs, on the other hand, have only been identified within *S. bulbocastanum* accessions which suggest that it is a recent gene on an evolution point of view (Van der Vossen et al., 2005, Vleeshouwers et al., 2008). Interestingly, *Rpi-blb2* is homologous to the tomato resistance gene *Mi-1* (Van der Vossen et al., 2005), which confers resistance against root-knot nematodes potato aphids and whitefly (Vos et al., 1998; Rossi et al., 1998, Nombela et al., 2003). Recently, the *Rpi-vnt1* gene family from *S.*

venturii has been identified on chromosome 9 (Foster et al., 2009). Three functional allelic variants have been cloned and comprise *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* (Pel et al., 2009). The *Rpi-vnt1* genes are promising, as they provide resistance towards many *P. infestans* isolates. Although at least two genotypes, *EC1* from Ecuador (Foster et al., 2009) and *NL08358* from the Netherlands, can overcome *Rpi-vnt1.1* in detached leave assays (Hein et al., unpublished data). *Rpi-vnt1.1* and *Rpi-vnt1.3* efficacy against *P. infestans* has been functionally confirmed through potato and tomato transformation (Pel et al., 2009). Despite the occurrence of a 14 amino acids insertion in the N-terminal region of *Rpi-vnt1.3* compared to *Rpi-vnt1.1*, they both share the same broad spectrum resistance (Pel et al., 2009). *Rpi-vnt1.2* differs from *Rpi-vnt1.3* in only one amino acid within this N-terminal insertion, but functional studies to confirm the importance of this change remain elusive. A functional homolog of *Rpi-vnt1* has been identified in *S. phureja* (Foster et al., 2009). Other *Rpi* genes have been identified from other *Solanum* species (Table I.2).

Table I.2: Overview of the *Rpi* genes identified so far from wild *Solanum* species. This table shows the *Solanum* species origin, the chromosomal location and the original reference corresponding to each gene.

Chr.	*R* gene	*Solanum sp.*	Reference
IV	*R2*	*S. demissum*	Li et al. 1998
	R2-like	*Non S. demissum*	Lokossou et al. 2009
	Rpi-edn1.1	*S. edinense*	Champouret, 2010
	Rpi-snk1.1	*S. schenckii*	Champouret, 2010
	Rpi-snk1.2	*S. schenckii*	Champouret, 2010
	Rpi-hjt1.1	*S. hjertingii*	Champouret, 2010
	Rpi-hjt1.2	*S. hjertingii*	Champouret, 2010
	Rpi-hjt1.3	*S. hjertingii*	Champouret, 2010
	Rpi-dmsf1	*S. demissum*	Hein et al. 2007
	Rpi-blb3	*S. bulbocastanum*	Lokossou et al. 2009
	Rpi-abpt	*S. bulbocastanum*	Lokossou et al. 2009
	Rpi-mcd1	*S. microdontum*	Sandbrink et al. 2000, Tan et al. 2008
	Rpi-bst1	*S. brachistotrichium*	Jones and Chu (communication Hein et al. 2009)
V	*R1*	*S. demissum*	El Kharbotly et al. 1994;
VI	*Rpi-blb2*	*S. bulbocastanum*	Vossen et al. 2003 and 2005
VII	*Rpi-pnt1*	*S. pinnatisectum*	Kuhl et al. 2001
	Rpi-mch1	*S. michoacanum*	Sliwka et al., 2012
VIII	*RB/Rpi-blb1*	*S. bulbocastanum*	Naess et al. 2000/Park et al. 2005
	Rpi-pta1	*S. papita*	Vleeshouwers et al. 2008
	Rpi-sto1	*S. stoloniferum*	Vleeshouwers et al. 2009
	Rpi-plt1	*S. polytrichon*	Wang et al. 2008

Chr.	*R* gene	*Solanum sp.*	Reference
IX	*Rpi-dlc1*	*S. dulcamara*	Golas et al. 2010
	Rpi-vnt1.1	*S. venturii*	Pel et al. 2009; Foster et al. 2009
	Rpi-vnt1.2	*S. venturii*	Pel et al. 2009; Foster et al. 2009
	Rpi-vnt1.3	*S. venturii*	Pel et al. 2009; Foster et al. 2009
	Rpi-mcq1.1	*S. mochiquense*	Smilde et al. 2005
	Rpi-mcq1.2	*S. mochiquense*	Smilde et al. 2005
	Rpi-edn2	*S. edinense*	Verzaux, 2010
	Rpi-ver1	*S. verrucosum*	Jacobs et al. 2010
	R8	*S.demissum*	Jo et al. 2011
	R1-like	*S. caripense*	Trognitz and Trognitz, 2005
	Rpi-phu1	*S. phureja*	Sliwka et al. 2006
	R9a	*S.demissum*	Dr. Jack Vossen personal communication (2016)
X	*Rpi-ber1*	*S. berthaultii*	Rauscher et al. 2006 ; Park et al. 2009
	Rpi-ber2	*S. berthaultii*	Rauscher et al. 2006 ; Park et al. 2009
	Rpi-dlc2	*S. dulcamara*	Golas et al., 2013
XI	*R3a*	*S. demissum*	Huang et al. 2005
	R3b	*S. demissum*	Huang et al. 2004
	R5	*S. demissum*	Huang et al. 2005
	R6	*S. demissum*	Huang et al. 2005
	R7	*S. demissum*	El Kharbotly et al. 1996
	R10	*S. demissum*	El Kharbotly et al. 1996
	R11	*S. demissum*	Bradshaw et al. 2006
	Rpi-pcs	*S. paucissectum*	Villamon et al. 2005
	R4	*S. demissum*	van Poppel et al., 2008
	Rpi-cap1	*S. capsicibaccatum*	Jacobs et al. 2010
	Rpi-sto2	*S. stoloniferum*	Champouret, 2010

All the *Rpi* genes cloned so far are members of the CC-NB-LRR family. Following the release of the potato genome, 755 putative NB-LRR genes have been annotated (Jupe et al., 2013).

This study has shown that the largest NB-LRR gene containing clusters correlate with the R2 cluster on chromosome 4 and the R3 cluster on chromosome 11. The study conducted by Jupe et al (2012; 2013) will help in understanding potato *R* gene evolution, and also in the cloning of other functional *R* genes from related wild *Solanum* species. It is also know now that 39.4% of the NB-LRR are pseudogenes (Potato Genome Sequencing Consortium, 2011), which may explain how plants can respond to the rapid evolution of *P. infestans* effectors (Haas et al., 2009). Indeed, it shows evidence of the rapid loss and gain of functional *R* genes.

2. *Solanum lycopersicum* or tomato

A. Origin and history of tomato

Solanum lycopersicum, commonly known as tomato, is part of the *Solanaceae* family and also belongs to the genus *Solanum*. The diversity of tomato is significantly reduced in comparison to potato and only nine diploid (2n = 2x = 24) wild species are recognised (Rick, 1978). Due to a number of qualities such as its diploid genome, inbreeding capacity, short life cycle, a rather small genome of 760 Mb spread over 12 chromosomes (Tomato Genome Consortium, 2012) and a great reproductive potential, tomato is a good model plant for *Solanaceae* research. Since 1992, a detailed genetic linkage has been available (Tanksley et al., 1992). However, the release of the tomato genome in 2012 (TGC, 2012) gave an invaluable tool for the cloning of new functional *R* genes and the understanding of their evolution. As in potato, similar annotation to predict tomato NB-LRR genes has been performed (Andolfo et al., 2013; 2014). Andolfo et al. (2013) also analysed the synteny between potato and tomato chromosomes, facilitating comparative analyses between both *Solanum* species.

Tomato originates from South America. Although the exact time of domestication is not known, tomato was already cultivated in Mexico when it was invaded by the Spanish in 1523 (Rick, 1978). Tomato was then introduced into Europe by Spanish conquistadors. It was later introduced to North America by immigrants in the 1600s (Rick, 1978). First believed to be

poisonous, it mostly spread in Europe as an ornamental and medicinal plant (Rick, 1978). The poisonous reputation of tomato persisted until the 1800s in many parts of the world (Rick, 1978). Tomato finally gained widespread popularity, especially in the early 20th century. Indeed, this fruit has become one of the most widely grown vegetables. Tomato has evolved to survive in various environmental conditions (Rice et al.,1987) and is grown all around the world, often in glasshouses when outdoor production is not possible due to cold temperatures (Foolad et al., 2008). Tomato is the second most consumed edible plant in the world after potato (Foolad et al., 2008). It is estimated that 4.6 million hectares of tomatoes are grown annually worldwide, producing more than 126 million tonnes (FAOSTAT). This highlights the economic importance of this crop. Even if tomatoes do not rank high in nutritional value, they contribute significantly to the dietary intake of vitamins A and C as well as essential minerals and other nutrients. Moreover, tomatoes are not only used for the fresh market but also for processed food and pharmaceutical products.

B. Resistance genes active against *Phytophthora infestans* in tomato

Similarly to potato, tomato production is threatened by *P. infestans*, provoking significant losses (Foolad et al., 2008). Somewhat similar to most potato cultivars, currently grown commercial tomato cultivars are not resistant to *P. infestans*. For disease control, growers employ crop rotations and frequent fungicide applications, as only very few known *P. infestans* race-specific resistance genes have been described in tomato. This is mainly due to *P. infestans* being a problem in potato until the 1990s, as many of the isolates infecting potato were not pathogenic to tomato (Nowicki et al., 2012). However, an increase in tomato *P. infestans* isolates has been observed and late blight has become the most devastating tomato pathogen (Foolad et al., 2008). This explains the increasing urgency in finding resistance to *P. infestans* to control the disease

Rpi-Ph1 has been identified from the *S. pimpinellifolium* accessions West Virginia 19 and 731 (Bonde and Murphy, 1952; Gallegly and Marvel, 1955) and has been mapped to tomato

chromosome 7 (Peirce, 1971). The resistance conferred by this gene is easily overcome by aggressive isolates and is thus not considered sufficient to control *P. infestans* (Foolad et al., 2008). *Rpi-Ph2* is an incompletely dominant resistance gene located on tomato chromosome 10 (Moreau et al., 1998). This resistance originates from the *S. pimpinellifolium* accession West Virginia 700 (Gallegly and Marvel, 1955) and can only slow down rather than stop disease progression (Goodwin et al., 1995). *Rpi-Ph3* from the resistant accession *S. pimpinellifolium L3708* was mapped to tomato chromosome 9 (Chunwongse et al., 2002) and was recently cloned (Zhang et al., 2014). This gene provides resistance to a wide range of *P. infestans* isolates which overcome *Rpi-Ph1* and *Rpi-Ph2*. However, the resistance carried in *S. pimpinellifolium L3708* is not only provided by *Rpi-Ph3*, but depends on the presence of yet undetermined hypostatic gene(s) (Kim and Mutscher, 2005). In another accession of *S. pimpinellifolium* called *L3707*, two independent genes have been identified (a partially dominant and a dominant epistatic gene) and further studies are required to demonstrate if either corresponds to *Rpi-Ph3* and related genes or not (Irzhansky and Cohen, 2006). Interestingly, *Rpi-Ph3* shares a high sequence identity ranging 74.7–78.7 % (Zhang et al., 2014) with several potato *R* genes, including *Rpi-vnt1.1* from *S. venturii*, *Rpi-mcq1* from *S. mochiquense* and *R9a* from *S. demissum* (Foster et al. 2009; Pel et al. 2009; Jones et al. 2009; Jack Vossen personal communication). Several quantitative resistance genes from wild tomato species have also been deployed in tomato cultivars, but none confers resistance to all the pathogen lineages found in the field (Foolad *et al.* 2008).

IV. Exploiting the advances in genome sequencing to identify new *Rpi* genes

1. Established method of identifying new resistance locus

The accepted start-point for studying a new resistance is to create a segregating population following a cross with a susceptible parent and phenotyping the progeny. Once phenotyped for the resistance of interest, the progeny are grouped into two bulks: a susceptible and a

resistant. This technique, referred to as Bulk Segregant Analysis (BSA), was first described for lettuce populations segregating for mildew resistance (Michelmore et al., 1991). The markers differing between the two bulks can be genetically linked to the pathogen resistance locus (Michelmore et al., 1991). However, BSA is not exclusive to pathogen resistance studies, and other traits such as drought resistance (Quarrie et al., 1999) can also be studied with this method. The obvious advantage of BSA, compared to analysing individuals of a given segregating population, is the small bulk size (Michelmore et al., 1991).

There are three major techniques used for genotyping once bulks are characterised. Restriction Fragment Length Polymorphism (RFLP) and Amplified Fragment Length Polymorphism (AFLP) are based on nucleotide polymorphism. RFLP is based on enzymatic restriction digestion, whereas AFLP uses PCR amplification as a mean to reveal the nucleotide polymorphisms. There have been numerous successful *Rpi* genes mapped in the past with both methods. Indeed, to site only few, R1, R6 and R7 were mapped using RFLPs (el-Kharbotly et al., 1994) while R2, R3a, R10 and R11 were mapped using AFLPs (Huang et al., 2005; Li et al., 1998; Bradshaw et al., 2006a). Although these techniques have proven to be efficient, recent advances in genome annotation and sequencing made room for faster targeted techniques. Indeed, a more recent approach for the genotyping of a BSA is based on a targeted analysis of the conserved NB-domain of NB-LRR genes, through PCR and BLAST searches (van der Linden et al., 2004). This technique, called NBS profiling is designed for small segregating populations and allows small bulks of three to ten progeny (Jacobs et al., 2010). Rpi-ver1 (Jacobs et al., 2010) and R8 (Jo et al., 2011) are two examples of genes mapped using NBS profiling.

2. Resistance genes in the era of next generation sequencing

A major shift in the way resistances to pathogens are studied was observed from 2010, when sequencing of whole genomes became much more accessible. The potato genome was published in 2011 (PGSC), shortly followed by the tomato genome (TGC, 2012). These

genomes provide an invaluable resource in identifying genes coding for important traits such as disease resistance. However, a resulting key challenge is the annotation of these genomes (Yandell and Ence, 2012) so their full potential can be used in functional and comparative analysis. When only a fraction of the genome is of interest, targeted enrichment has proven to be efficient in limiting the complexity of the data, while providing a better read depth (Cronn et al., 2012). In the case of finding new resistances, this concept has been exploited and resulted in the development of RenSeq (Resistance enriched Sequencing) (Jupe et al., 2013; 2014). Using this method, Jupe et al. (2013) were able to annotate a further 317 candidate potato NB-LRR genes, compared to the previous annotation of the DM genome (Jupe et al., 2012). Renseq is based on the sequencing and analysis of NB-LRR-enriched samples, comprising a parent resistant and a parent susceptible, together with a bulk of resistant and a bulk of susceptible progeny. The enrichment is made using probes specifically designed to capture NB-LRR sequences (Jupe et al., 2014). In Figure I.6, the workflow of RenSeq is illustrated.

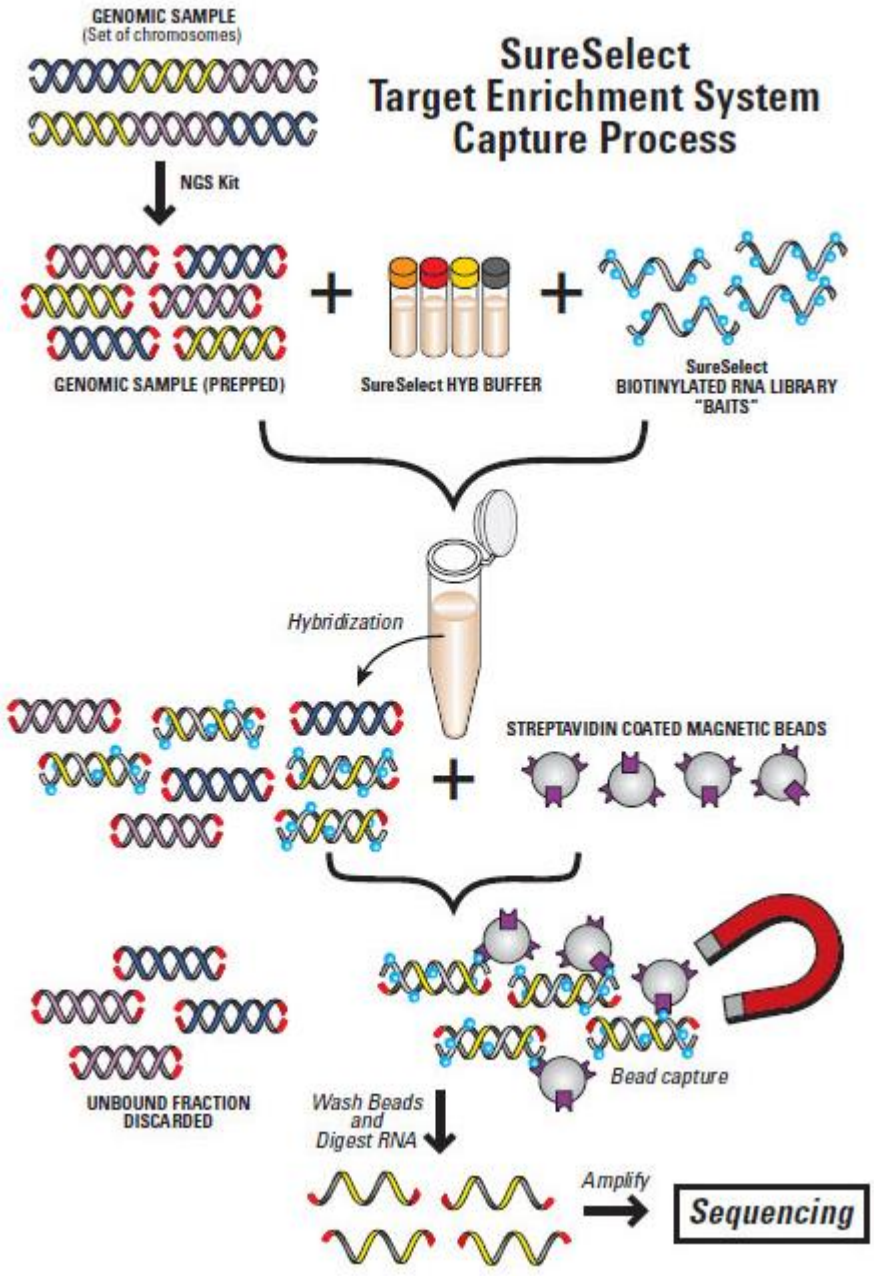

Figure I.6: Workflow depicting the different steps of a RenSeq. This figure is taken from Agilent (http://www.genomics.agilent.com/article.jsp?pageId=3083).

V. Scope of this thesis

In the recent years, Next Generetion Sequencing has completely revolutionised the study of resistance genes. Considering the recent advances in tomato and potato NB-LRR annotations, the fist aim of this project was to develop a new NB-LRRs probe library, more representative of the current knowledge. The objective was also to establish a better diagnostic tool for

known *Rpi* genes, so resistant material can be more rapidly assessed for novelty in breeding programs. On the pathogen side of the interaction, the aim was to identify the *Avr-Ph3*, effector recognised by the *Ph3* tomato resistance against *P. infestans*.

Chapter III focuses on the creation of the new NB-LRRs probe library, which will be used in a RenSeq analysis of a diploid potato population B3C1HP, which segregates 1:1 for late blight resistance. The focus point of this study being *Rpi-Ph3,* the probe library will be tested *de novo* to evaluate if it would have enabled the identification of *Rpi-Ph3* and homologous sequences. The *Rpi-Ph3* published sequence will also be used to develop PCR markers, in order to facilitate breeding.

In chapter IV, an 'omics'-based approach for the screening of the CPC to prioritise novel *Rpi* will be developed, and tested on a characterised CPC accession of *S. okadae*. Once established, the technique will also be applied to characterise the B3C1HP potato population (Li et al., 2015) detailed in Chapter III.

Chapter V focuses on the identification of Avr-Ph3. The first objective will be to establish a robust, transient expression system of effectors to screen an association panel of tomato lines. Candidate *Avr-Ph3* genes will then be confirmed by co-infiltration with the cloned *Rpi-Ph3* resistance gene in *N. benthamiana*. Lastly, the role of *Avr-Ph3* candidates in *P. infestans* pathogenicity will be assessed upon transient expression of the candidates in *N. benthamiana*.

The overall findings of this PhD will be discussed in the final chapter VI, together with the impacts this work can have on future research and late blight management in the fields.

Chapter II: Material and Methods

I. Plant and pathogen material

1. Plant material

A. Potato material:

The James Hutton Institute is hosting a collection of wild potato species originating from Mexico and South America. It is composed of more than 1,800 potato accessions spread over more than 80 species, collected from several expeditions since the 1930s. In this thesis, the focus was on diploid species, in order to simplify the background in which resistances were studied. A total of 125 accessions representing 34 diploid species were screened in the course of this project (Table IV.1).

The different accessions were maintained in the glasshouse by Brian Harrower (JHI) through regular cuttings. The glasshouse was set for 18H of light at 16°C during the day and 12°C in the dark for 6H at night, and plants of about one month old were used in the different screenings. The different accessions were backed up through *in vitro* culture of cuttings with the help of Brian Harrower. When available, tubers of the different accessions are also stored at 4°C, in the case any of them was to not be successful in the glasshouse or the *in vitro* culture.

B. Tomato material

An association panel in tomato was provided by Dr. Véronique Lefebvre and René Damidaux, from INRA Avignon, France. This association panel consisted of eight susceptible, six *Rpi-Ph2* and three *Rpi-Ph3* lines (Table II.1). As the study developed, additional *Rpi-Ph3* lines were requested, in order to obtain a more robust set of plants for *Rpi-Ph3*. Dr. Véronique Lefebvre and René Damidaux were able to send another two lines of *Rpi-Ph3* tomatoes (Table II.1).

Table II.1: List of the 19 tomato lines forming the association panel. Their resistance specificity is described. Written in grey are the *Rpi-Ph3* tomato lines provided later in the study.

Susceptible	Resistant	
	Rpi-Ph2	***Rpi-Ph3***
Heinz 1706	Héline	LA1269
Earlymech	Mecline	LA1269 (RZ)
Europeel	Piline	L3707/25/09
Floradade	Fline	L3708
Pieralbo	Piéraline	L3708/22/09
WVA106	WVA 63	
Monalbo		
Moneymaker		

The tomato plants were grown from seeds in the glasshouse, with a 16H day period at 24°C and an 8H night period at 17°C. Plants were screened at about one month old. Some plants were kept until flowering and the production of fruits, in order to collect more seeds. The different tomato lines were also kept *in vitro* culture as a back-up.

Seeds of the different lines were surface sterilized, using the following protocol: 15min under continuously running tap water (Ishag et al., 2009), rinse with sterile distilled water (SDW), soak 15min into a ±0.50% sodium hypochlorite solution (e.g. 15% Clorox® solution) (Ishag et al., 2009; Chaudhry et al., 2010), rinse 3 times with SDW, soak 15-20s into 80% ethanol (Chaudhry et al., 2010) and rinse with SDW. This procedure was carried out under sterile conditions in a laminar flow hood cleaned with 75% ethanol. The seeds were grown *in vitro* on half strength Murashige and Skoog Medium (Murashige and Skoog, 1962) supplemented with 30g/L Sucrose. Two different brands of agar were tested: BACTO and MELFORD, but MELFORD gave the best growth. Around 50mL media per Magenta® GA-7 vessels was poured. Five seeds were placed into a vessel, in duplicates. Vessels were closed with vented

lids. The vessels were stored at 21°C, in the dark during the first four days after the sterilisation, then at 16H light per day.

C. *Nicotiana benthamiana* (*N. benthamiana*)

N. benthamiana plants were grown and maintained by the glasshouse staff of the JHI. Plants were grown at 22°C with a day period of 16H and 18°C night period of 8H. Plants were screened at about 1 month old.

2. Late blight material

The different isolates of *P. infestans* (Table II.2) were established *in vivo* on leaves of the late blight susceptible *cv.* Craig's Royal, and passaged by Brian Harrower (JHI) through several generations, according to Andrivon et al., 2011. Their genotype, mating type and race specificity are described in Table II.2, when known.

Table II.2: Late blight isolates used in the screening of *S. okadae* accessions. Shown is their name, genotype, mating type and race specificity where known.

***P. infestans* isolate**	**Genotype**	**Mating type**	**Race**
2009_7654A	13-A2_78	A2	R1-7, R10, R11
2010_7822B	6-A1	A1	R1, R3, R4, R7, R10, R11
2010_7814A	23-A1	A1	R1, R3, R4, R7
2010_8122D	8-2-A1	A1	R1, R3, R4, R7, R10, R11
2010_7838A	Misc	A1	R3, R4, R7, R11
EC1	uncharacterised	uncharacterised	Rpi-vnt1.1

II. Transient expression assays

1. *S. okadae* effector screen

All the *P. infestans* effectors (Table IV.3) were cloned into the binary vector pGRAB and transformed into the *A. tumefaciens* strain Agl1 with VirG and pSOUP. An empty vector was

used as a negative control. Infiltrations and analysis of infiltration sites were conducted as described in Gilroy et al. (2011), with the help of Dr Xinwei Chen and Gaëtan Thilliez (JHI).

2. Tomato effector screens

A. Identification of a suitable transient expression system in tomato

a. Agro-infiltration of different *Agrobacterium tumefaciens* strains

i. Agro-infiltration of Agl1

Three negative controls were tested using the *Agrobacterium tumefaciens* strain Agl1, containing pSOUP and VirG. The empty pGRAB (pGRAB:empty), pGRAB expressing red fluorescent protein tdTomato (tdT) and *P. infestans* Inf1 that is typically recognised in *Nicotiana* species but not tomato with this system. The constructs preparation and infiltrations were performed according to Gilroy et al. (2011), on one month old Moneymaker plants. Three different optical densities measured at 600nm (OD_{600}) have been tested and include 0.1, 0.3 and 0.5.

ii. Agro-infiltration of LBA4404

The *A. tumefaciens* strain LBA4404, kindly provided by the Huitema lab, was tested with non-transformed bacteria, at different OD_{600} ranging between 0.1 and 1, on one month old Moneymaker plants. The constructs preparation and infiltrations were performed according to Gilroy et al. (2011).

iii. Agro-infiltration of 1D1249

The *A. tumefaciens* strain 1D1249, kindly provided by the Huitema lab, was tested with two different GFP constructs, pb7:WGF2 and pb7:03192. Infiltrations were performed at OD_{600} of 0.1, 0.2 and 0.5, on one month old Moneymaker plants. The constructs preparation and infiltrations were performed according to Gilroy et al. (2011).

b. Virus based agro-infiltration

Virus based effector expression was also considered as an option (Liu et al, 2002; Faivre-Rampant and Gilroy et al, 2004). Recombinant Tobacco rattle virus (TRV) and Potato Virus X (PVX) delivered via agro-infiltration with Agl1

i. TRV

TRV-based delivery was investigated with two constructs, TRV:GFP and TRV:CRN8 (a crinkling inducing protein from *P. capsici*), which were tested at an OD_{600} of 0.01, 0.05 and 0.1, on one month old Moneymaker plants. Those constructs were kindly provided by the Huitema lab (University of Dundee and JHI). The constructs preparation and infiltrations were performed according to Gilroy et al. (2011).

ii. PVX

PVX-based delivery was investigated through the infiltration of six constructs, in the *A. tumefaciens* strain GV3101 containing the PVX expression vector pGR106. Those constructs included Inf1, a truncated and non-functional green fluorescent protein (called αGFP), CRN1 and CRN2 which are two cell death inducers from *P. capsici*, and two variants of CRN2. The construct 10F is a truncated, still functional and able to induce cell death, variant of CRN2; whereas the variant 11F is a non-functional truncation of CRN2. Three different OD_{600} have been tested and include 0.1, 0.05 and 0.01. Constructs were grown in 50mL tubes with 5mL of YEB medium (prepared by the JHI media kitchen facility) supplemented with the appropriate antibiotics, for 24H from plate colonies, at 27°C and 250rpm. The agro-suspension was prepared as described in Gilroy et al. (2011). The agro-infiltrations were performed using a syringe without a needle to pressure infiltrate the bacterial suspension into a defined area of the abaxial leaf, according to Vleeshouwers et al (2011). Phenotypic responses were scored at 8dpi, under normal light and UV light.

iii. Tooth pick inoculation with PVX

Tooth pick inoculations of PVX constructs were investigated in different tomato lines, as described in Vleeshouwers at al. (2008).

B. Effector screen using the GV3101-PVX system

The preliminary experiments showed that the *Agrobacterium tumefaciens* strain GV3101 containing the pGR106 vector (binary PVX) was the most efficient delivery system in tomato, and was used for the tomato effector screen. A library of 96 *Phytophthora infestans* effectors were re-cloned into this system by Dr. Sophie Mantelin (JHI) (Table IV.3) and infiltrated in the tomato association panel, as described previously. The truncated, non-functional GFP and the cell death inducer CRN2 were infiltrated as negative and positive control respectively, on every leaf infiltrated. The OD_{600} of the bacterial suspension was adjusted to 0.05. Phenotypic responses were scored at 8 days post infiltration (dpi), and pictures were taken under normal and UV light. An arbitrary scale from 0 (no phenotypic response) to 4 (really strong HR) was used to score the infiltrated sites.

Table II.3: List of the 96 effectors and effector candidates cloned into GV3101-PVX by Dr. Sophie Mantelin (JHI) and used in the tomato effector screen. # is the abbreviation

number used for the PITG identifier. If known, the AVR function of individual RXLR effectors is specified in brackets.

#	Effector name	#	Effector name	#	Effector name
1	PITG_00366	33	PITG_10232	65	PITG_20301
2	PITG_00582	34	PITG_10540	66	PITG_20303 (Avr-blb2)
3	PITG_00821	35	PITG_10654	67	PITG_21388 (IpiO1)
4	PITG_02860	36	PITG_11484 (Avr10)	68	PITG_21388 (IpiO4)
5	PITG_03192	37	PITG_11507	69	PITG_21740
6	PITG_04085	38	PITG_12731	70	PITG_22604
7	PITG_04089	39	PITG_12737	71	PITG_22724
8	PITG_04090 (Avr-blb2 family)	40	PITG_13093	72	PITG_22798
9	PITG_04097	41	PITG_13628	73	PITG_22804
10	PITG_04145 #2	42	PITG_13959	74	PITG_22922
11	PITG_04145 #4	43	PITG_14371 (Avr3a^{EM})	75	PITG_23015
12	PITG_04266	44	PITG_14371 (Avr3a^{KI})	76	PITG_23226
13	PITG_04314	45	PITG_14443	77	PITG_23239
14	PITG_04339	46	PITG_14736	78	PITG_05096
15	PITG_04388	47	PITG_15110	79	PITG_05846
16	PITG_05750	48	PITG_15278	80	PITG_08278
17	PITG_06087	49	PITG_15287	81	PITG_10673
18	PITG_06099	50	PITG_15972 (Avr2 family)	82	PITG_11383
19	PITG_06308	51	PITG_16195	83	PITG_13625
20	PITG_06478	52	PITG_16240	84	PITG_14783
21	PITG_07550 #1	53	PITG_16294 (Avr-vnt1)	85	PITG_14833
22	PITG_07550 #8	54	PITG_16427	86	PITG_15123
23	PITG_07550 #9	55	PITG_16705	87	PITG_15125
24	PITG_07689	56	PITG_16726	88	PITG_15127
25	PITG_08599	57	PITG_16737	89	PITG_16663
26	PITG_09216	58	PITG_17063	90	PITG_17309
27	PITG_09218	59	PITG_18215	91	PITG_17316#1
28	PITG_09585	60	PITG_18221	92	PITG_17316#2
29	PITG_09680	61	PITG_19617	93	PITG_18670
30	PITG_09732 #1	62	PITG_19800	94	PITG_21778
31	PITG_09732 #2	63	PITG_19942	95	PITG_22870 Avr2
32	PITG_09732 #3	64	PITG_20300 (Avr-blb2 family)	96	PITG_22870 (Avr2-like)

3. *N. benthamiana* co-infiltrations

The gene *Rpi-Ph3* has been published (Zhang et al, 2014) and collaboration with the authors allowed access to the construct in the *Agrobacterium tumefaciens* strain Agl1 with virG, in

the vector pK7WG2 (35S promoter). Co-infiltrations between *Rpi-Ph3* and all the effectors available (see Table IV.5) were performed in *N. benthamiana*. Infiltrations were performed as explained previously, with an OD_{600} of 0.5 at a 1:1 ratio and with P19 at a final OD_{600} of 0.01. The 96 effectors were tested in at least three independent replicates, with two infiltration sites per replicate. However, the *Avr-Ph3* candidates identified in the effector screen (Figures V.3 and V.4) were tested a further 2 independent replicates. Results were scored at 8dpi and pictures were taken under normal and UV light. An arbitrary scale from 0 (no phenotypic response) to 4 (strong HR) was used. Controls including the different effectors and *Rpi-Ph3* infiltrated alone, as well as *R3a* and $Avr3a^{KI}$, were infiltrated in parallel of the co-infiltrations. The co-infiltration of *R3a* and $Avr3a^{KI}$ was used as a positive control (Armstrong et al., 2005).

III. Pathogen assays

1. Late blight screens

Detached leaf tests were carried out as described by Whisson et al. (2007) and seedling and whole plant tests (2 replicates) as described by Stewart et al. (1983) and Bradshaw et al. (2006) respectively. Disease was scored between 5 and 8 days post infection, on a scale of resistance ranging from 1 = very susceptible to 5 = very resistant for seedling and detached leaf tests and 1 = very susceptible to 9 = very resistant; symptomless plants, for whole plants according to the Malcolmson scale (Cruickshank et al., 1982). The whole screen described in Chapter IV was a joint effort with Brian Harrower, James Lynott, Dr Alison Lees and Gaynor McKenzie (JHI).

2. Gain of pathogenicity assays

Infiltrated constructs were in the *A. tumefaciens* strain Agl1, in the vector pGRAB, with VirG and pSOUP, and prepared as described in Gilroy et al. (2011). Infiltrations were performed at OD_{600} 0.1, with OD_{600} 0.05 of P19. Infiltration sites were inoculated the following day with

10µL droplets of *P. infestans* tdT-88069 zoospore suspension. Late blight lesions were measured at 7dpi.

The zoospore suspension was prepared from a plate culture of tdT-88069, according to a protocol elaborated by Dr. Hazel McLellan (JHI). The plate was scraped with cold (from the fridge) 10mL of SDW. The suspension was collected in a 50mL tube, and centrifuged for 15min, at 2750rpm and 4°C. The pellet was re-suspended in 3mL of fresh SDW. The sporangia concentration was adjusted to 50,000 sporangia/mL and the suspension left for at least 3H in the fridge, before the inoculation could be performed.

The Trypan Blue staining was performed according to a protocol provided by Dr. Ingo Hein. Each leaf was placed in a 50mL Falcon tube, and submerged in a Trypan Blue stain (Tryan Blue, SDW, Phenol, Lactic acid and Glycerol at 1:1:1:1:1 ratio). The samples were boiled for 2min, then left to cool so they can be handled. The Trypan Blue was poured out of the tubes and leaves were rinsed with SDW. The leaves were then submerged in a Chloral Hydrate solution (5:2 ratio) and left to de-stain overnight. The Chloral hydrate solution was then changed and leaves left to de-stain further. Once sufficiently de-stained, pictures of the leaves were taken on a light table, in a Petri dish with SDW.

IV. Molecular assays

1. PCR based analyses

A. *Rpi-vnt1* PCR screen and allele mining

a. *Rpi-vnt1* specific PCR screen

The resistance gene *Rpi-vnt1* has three functionally equivalent: *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3*, which are known to share more than 98% sequence homology (Pel et al., 2009; Forster et al., 2009). Primers were designed using the *Rpi-vnt1.1* sequence in Primer 3. A set of full length primers, called Rpi-vnt1_Full (Table II.4), was designed so the full length *Rpi-*

vnt1.1 (2676bp long), *Rpi-vnt1.2* and *Rpi-vnt1.3* (2718bp long), as well as their variants would be amplified. Another set of primers, called Rpi-vnt1_diag (Table II.4), was also designed so a shorter (~500bp) portion of the gene would be amplified. Both primer sets were tested on the *Rpi-vnt1* transformed potato *cv.* Desirée and water controls. All the PCRs were performed using the Q5 polymerase (New England BioLabs) according to the manufacturer protocol. The PCR program settings specific to each primers sets is described in Table II.4. PCR products were loaded in a 1% agarose gel supplemented with 0.5µg/mL of ethidium bromide.

Table II.4: Sequences of the two sets of primers specific to *Rpi-vnt1*. The primer dependant settings of the PCR program (using the protocol of the Q5 polymerase from New England BioLabs) are specified.

Primer name	Primer sequence	PCR program specificities	
		Annealing	Elongation
Rpi-vnt1_Full_F_130	ATGAATTATTGTGTTTACAAGACTTGG	63°C for 20s	72°C for 1min15s
Rpi-vnt1_Full_R_135	TTATAGTACCTGTGATATTCTCAACTTTGC		
Rpi-vnt1_diag_F_257	CTGACGGAAGAGGAAAGGAA	55°C for 20s	72°C for 15s
Rpi-vnt1_diag_R_85	ATACTCTCAAGTACTCTGTTC		

b. *Rpi-vnt1* allele mining of the *S. okadae* accessions

Rpi-vnt1-like genes have been amplified from the *S. okadae* accessions 7129, 7625 and 7629 through PCRs with the *Rpi-vnt1* specific primers Rpi-vnt1_Full (Table II.4). To assess the diversity of the *Rpi-vnt1-like* sequences PCR products were cloned into the vector pGEM-T easy for Sanger sequencing, according to the manufacturer's recommendations (pGEM®-T Easy Vector System - Promega). Recombinant clones were selected following transformation of the constructs into electro competent *Escherichia coli* DH10B and DH5α cells (Invitrogen) using colony PCR with the gene specific primers mentioned above. Sequencing products were subjected to a BLASTn analysis and compared to functional *Rpi-vnt1* variants (Pel et al., 2009) using Geneious v5.6.3 (Biomatters).

B. Development of *Rpi-Ph3* PCR markers

The published sequence of *Rpi-Ph3* (Zhang et al, 2014) was used to design two sets of primers specific to *Rpi-Ph3*. A BLAST of *Rpi-Ph3* (Yuling Bai personal communication) against the tomato genome (Jupe et al., 2013) was performed to identify closely related but non-functional genes present in the susceptible tomato Heinz 1706. The cloned potato genes *Rpi-vnt1.1* from *S. venturii* (Foster et al., 2009, Pel et al., 2009) and *Rpi-mcq1* from *S. mochiquense* (Jones et al., 2009), as well as he *Tm-2²* from tobacco (Lanfermeijer et al., 2003), share a high identity with *Rpi-Ph3* (Zhang et al., 2014). Those genes were aligned to the Heinz 1706 *Rpi-Ph3* paralogs identified in the BLAST and *Rpi-Ph3* using Geneious 6.1.8 (Biomatters). Regions specific to *Rpi-Ph3* were identified (Figure III.4) and used to design two sets of primers using Primer3 (Table II.5).

The primers described in Table II.5 were tested on DNA from all the tomato lines (eight susceptible lines, seven *Rpi-Ph2* lines and five *Rpi-Ph3* lines), as well as *N. benthamiana*, *N. sylvestris*, the peppers Californian Wonder and CM334, the *Rpi-vnt1* transformed potato *cv.* Desirée, and water. All the DNA extractions were performed using the DNeasy Plant Mini kit (QIAGEN) according to the manufacturer's protocol. DNA quality was checked with a 1% agarose gel supplemented with 0.5µg/mL of ethidium bromide, as well as through PCR with the 18S primers pair. All the PCRs were performed using the GoTaq Flexi DNA polymerase (PROMEGA) according to the manufacturer protocol. The PCR program settings specific to each primers sets is described in table 1. PCR products were loaded in a 1% agarose gel supplemented with 0.5µg/mL of ethidium bromide.

Table II.5: Sequences of the two sets of primers specific to *Rpi-Ph3*. The primer dependant settings of the PCR program (using the protocol of the GoTaq Flexi polymerase from PROMEGA) are specified.

Primer name	Primer sequence	PCR program specificities

		Annealing	Elongation
Rpi-Ph3_Full_F.2	ATGGCTGATATTCTTCTTACTGCAGTCG	65.5°C for 45s	72°C for 2min35s
Rpi-Ph3_Full_R.2	TCATACTCTCAGCTTTGCAAGACGTTT		
Rpi-Ph3_diag_F.2	GTGTGTATGATAAGTTTGTTGAACAGAT	59°C for 45s	72°C for 18s
Rpi-Ph3_diag_R.2	TACATAACCCTCTACCATAATTAAGCG		

2. Western blot assays

Two different methods have been used, with a quick diagnostic method in use in the Huitema Lab (University of Dundee and JHI), and a more time consuming but cleaner method developed by Dr. Sophie Mantelin (JHI).

A. Quick diagnostic Western blot method

The protein extraction was performed from flash-frozen leaf discs of *N. benthamiana,* which were infiltrated at OD_{600}=0.5 with the corresponding constructs two days before. For each construct, two frozen leaf discs were placed into a 2mL Eppendorf. A metal bead was added to each sample and the tubes were placed into a tissue lyser for 1min at a frequency of 30. Two volumes of loading buffer (0.25M of Tris pH=6.8 with 6% SDS, 40% glycerol, 0.04% bromophenicol blue, 20mM DTT and SDW) were then added to each sample. The tubes were boiled for 5min at 95°C, then centrifuged at 13,000rpm for 30s. 10µL of the supernatant of each sample was directly loaded in a precast gel, alongside the PageRuler ladder (ThermoFischer). The gel was run at 120V for the first quarter of the migration, then 200V until the end of the migration. A Trans-Blot Turbo Transfer System pack (BioRad) was then used according to the manufacturer's protocol, and revelation was performed in the trans-blot turbo (BioRad) for 7min.

Following the transfer, the membrane was blocked with a 30min incubation of 1X Phosphate Buffered Saline (called PBS = 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM $KH_2PO_4(H_2O)_{12}$, pH 7.4) supplemented with 0.1% Tween-20 (then called PBST) and 5% non-

fat dry milk, on a rotary shaker at room temperature. The immune-detection for GFP was performed overnight at 4°C on a rotary shaker, with an anti-GFP mouse antibody in the same PBST+milk buffer described before. The secondary antibody anti-mouse was incubated in the same buffer, for 1H at room temperature on a rotary shaker. The membrane was then rinsed four times for 5min in the PBST+milk buffer, at room temperature on a rotary shaker.

The chemiluminescence-detection of the secondary antibody conjugated with horseradish peroxidase was performed with 1:1 solution of SuperSignal West Femto (Pierce-Thermo Scientific reference 34095) and enhancer. The membrane was washed with this solution for 5min using a pipetman. The membrane was then placed in a G:Box (Syngene) for imaging.

The protein detection was performed by Coomassie Blue staining. The membrane was incubated at room temperature for 5min on a rotary shaker, and rinsed three times for 5min with SDW on a rotary shaker.

B. Traditional GTEN Western blot method

The protein extraction was performed from flash-frozen leaf discs of *N.benthamiana,* which were infiltrated at OD_{600}=0.5 with the corresponding constructs two days before. For each construct, two frozen leaf discs were placed into a 1.5mL Eppendorf and ground in liquid nitrogen using a pestle. 200µL of extraction buffer were added and the tube was vortexed several times quickly, before being put on ice to incubate for 20min. The extraction buffer consists of GTEN buffer supplemented with 0.15% Nonidet P-40, 2% polyvinylpolypyrolidone, 10mM dithiothreitol (DTT), 1mM phenylmethylsulfonyl fluoride and 1X protease inhibitors (Sigma reference P9599). The GTEN buffer is prepared with SDW supplemented with 10% glycerol, 25mM tris hydrochloride, 1mM ethylenediaminetetraacetic acid and 150mM sodium chloride. After the incubation on ice, tubes were vortexed briefly and centrifuged for 1min at 4°C at 10,000rpm. The supernatants were transferred in a new tube. This step of centrifugation and transfer of the supernatant was repeated another two times, to get rid of

any cell debris. In a new Eppendorf tube, 5.25µL of DTT 0.5M was added to 70µL of the supernatant, and 35µL of 4X loading buffer (Invitrogen LDS sample, reference NP0007). The samples were then boiled for 5min then put in ice for a few minutes. Samples were then centrifuged briefly before loaded (30µL) onto a pre-casted gel, alongside 6µL of PageRuler Plus prestained ladder (ThermoFischer). Migration was performed on ice at 180V. The transfer onto a membrane was then performed for 2H at 30V.

The protein detection was performed before the immune-detection, by Ponceau Red staining, with a 0.5% Ponceau Red and 1% Acetic acid solution. The membrane was incubated at room temperature for 5min on a rotary shaker, and rinsed twice with SDW.

The immune-detection for GFP was performed after blocking the membrane in a 1X PBST buffer (1X Phosphate Buffered Saline with 0.1% Tween-20) with 5% non-fat dry milk for 2.5H, on a rotary shaker at room temperature. The primary anti-body, a rabbit polyclonal serum anti-GFP (Santa Cruz reference sc-8334), was added in a 1:5,000 dilution to a 1X PBST buffer with 5% non-fat dry milk. The solution was poured onto the membrane and left overnight to incubate at 4°C on a rotary shaker. The membrane was then quickly rinsed with 1X PBST buffer, and washed three times in 1X PBST for 15min each, on a rotary shaker. The secondary antibody, a goat anti-rabbit polyclonal immunogen conjugated with horseradish peroxidase (Sigma reference A0545) was added in a 1:80,000 dilution to a 1X PBST buffer, and poured on the membrane for an hour incubation, at room temperature on a rotary shaker. The membrane was then quickly rinsed with 1X PBST buffer, and washed three times in 1X PBST for 15min each, on a rotary shaker. The membrane was rinsed one last time with 1X PBS for 5 min at room temperature on a rotary shaker.

The chemiluminescence-detection of the secondary antibody conjugated with horseradish peroxidase was performed with 1:1 solution of SuperSignal West Femto (Pierce-Thermo Scientific reference 34095) and enhancer. The membrane was washed with this solution for

5min using a pipetman. The excess detection reagent solution was drained off before the membrane was placed, protein side down, on a fresh piece of SaranWrap, which was then taped to a X-ray film cassette with the protein side up. A sheet of autoradiography was then placed on top of the film for 5 seconds, in a dark room using red safe light, and developed immediately.

3. Enrichment

RenSeq target enrichment and sequencing was performed with the help of Dr Miles Armstrong (JHI), according to Jupe et al., (2013; 2014), and with minor modifications. The covaris sonicator M220 (Covaris), was used for the fragmentation of DNA to approximately 500bp in length, with the following settings: 50W Peak Incident Power, 20% Duty Factor, 200 cycles per burst, 60 seconds treatment time and 50µL volume with 1µg starting amount. These settings were chosen after a trial run of three different conditions (Table II.6), in order to get the best possible output.

Table II.6: Different settings of the covaris sonicator M220 (Covaris) tested on the BS and BR of B3C1HP. Results of the sonication are shown in Table III.2.

Conditions	Peak power (W)	Duty factor	Cycles/burst	Treatment time (in s)
#1	75	10%	200	60
#2	50	20%	200	60
#3	75	20%	200	50

The fragments sizes were checked using a Bioanalyser (Agilent) and no upper size selection was conducted. The samples were quantified using Qubit (Thermofisher) and the enrichment was started with 750ng of indexed libraries. Added to the hybridization was 1µL of 1000mM universal blocking primer, containing 6 inosines in place of the 6 nucleotide index sequence and a 3' spacer C3 modification to prevent the primer from participating in any subsequent PCR amplification. The post capture amplification was performed with the Herculase II

polymerase (Agilent). Sequencing was conducted on an Illumina MiSeq platform using the 2x 300bp kit. The raw sequence reads were deposited at the European Nucleotide Archive under accession number PRJEB12834.

V. Bioinformatics and statistical analyses

1. Bioinformatics

A. Sequence analysis

All the *in silico* sequence analyses and visualisations were performed in Geneious R6 (v6.0 and 6.1) created by Biomatters. This software is available from http://www.geneious.com.

B. Probe library design

The new probe library was designed on the base of the new NB-LRRs annotations in potato and tomato (Jupe et al., 2013; Andolfo et al., 2014) and a list of known *R* genes (Jupe et al., 2013). The first step was to send the sequences of the 755 potato, 397 tomato and 53 cloned NB-LRRs to the Agilent SureDesign team (Agilent Technologies). A preliminary set of probes was returned, and checked for identical sequences using the tool “remove duplicate” in excel. Such probes were deleted and remaining probes were run through ReapeatMasker (http://www.repeatmasker.org/, Institute for Systems Biology), to identify probes hitting repetitive regions. The remaining probes were then BLASTed against the potato and tomato genome using a self-written script in Python. This analysis was performed in order to check for the probes specificity and probes hitting more than 80 genes were further studied. Such probes were BLASTed against nr using a self-written script in Python. Probes related to resistance genes were conserved in the design while the others were discarded. The remaining probes were checked for coverage of all the NB-LRRs used in the initial desing, using a self-written script in Python. Indeed, the aim of this new library was to have all the NB-LRRs covered by at least one probe.

C. (d)RenSeq analyses

The following computational analysis was performed by Dr Katie Baker (JHI). Paired-end Illumina MiSeq reads were first checked with FastQC (v0.10.0; Andrews, 2010) and then quality and adapter trimmed with cutadapt (v1.9; Martin, 2011) to a minimum length of 100bp and minimum base quality of 20. The trimmed reads were then mapped to the potato DM reference genome (v4.03; PGSC, 2011; Sharma et al., 2013) or a FASTA file containing twelve cloned R genes using Bowtie2 (v2.2.1; Langmead and Salzberg, 2012) in very-sensitive end-to-end mode.

The known R genes comprise: *R1* (GenBank: AF447489.1; Ballvora et al., 2002), *R2* (GenBank: FJ536325.1; Lokossou et al., 2009), *R2-like* (GenBank: FJ536323.1; Lokossou et al., 2009), *R3a* (GenBank: AY849382.1; Huang et al., 2005), *R3b* (GenBank: JF900492.1; Li et al., 2011), *Rpi-sto1* (GenBank: EU884421.1; Vleeshouwers et al., 2008), *Rpi-pta1* (GenBank: EU884422.1; Vleeshouwers et al., 2008), *Rpi-blb1* (GenBank: AY426259.1; van der Vossen et al., 2003), *Rpi-blb2* (GenBank: DQ122125.1; van der Vossen et al., 2005), *Rpi-blb3* (GenBank: FJ536346.1; Lokossou et al., 2010), *Rpi-abpt* (GenBank: FJ536324.1; Lokossou et al., 2009) and *Rpi-vnt1.1* (GenBank: FJ423044.1; Foster et al., 2009).

For read mapping, discordant and mixed mappings were disabled and maximum insert was set to 1000bp. Four score-min parameters were used in different mapping runs: “L,-0.03,-0.03”, “L,-0.06,-0.06”, “L,-0.3,-0.3” and “L,-0.6,-0.6”, approximately equal to 0.5%, 1%, 5% and 10% mismatch rates, respectively. The resulting BAM files were sorted and indexed using SAMtools (v0.1.18; Li et al., 2009).

The percentage of mapped reads on target was calculated as the proportion of reads mapping to an annotated, targeted RenSeq region in the DM genome reference. Intersecting these regions (+/-1000bp) against the mapped reads using BEDTools (v2.20.1; Quinlan and Hall, 2010) gave the number of on-target reads. The reads on target were then calculated as

a proportion of the total number of mapped reads. Read coverage to on target regions was estimated by dividing the number of base pairs mapped to the 704 *R* genes (+/-1000bp) on chromosomes 1 to 12 by their total length (plus 2000bp per gene). Read coverage was also estimated for the twelve R gene reference set by dividing the total number of mapped reads by the total length of the reference set.

2. Statistics

All the statistical analyses were performed in Genstat 17th Edition, using General Analysis of Variance. These ANOVA were performed under Multiple Comparisons, using Fisher's Protected least significant difference tests with a significance level of 0.05.

Chapter III: Design of a comprehensive NB-LRR bait library for potato and tomato, and *in silico* analysis of *Rpi-Ph2* and *Rpi-Ph3* resistances

In this chapter, a new NB-LRR probe library was designed to take into account the reannotation of both the potato and the tomato predicted NB-LRR genes (Jupe et al., 2013; Andolfo et al., 2014). This new library was designed to be as comprehensive as possible, given the advances made in both genomes in the recent years. The aim was originally to use the library on a *Rpi-Ph3* segregating population to fine map and clone the underlying resistance gene. Similar to *Rpi-Ph2* and despite being used in tomato breeding, little was known about these resistance genes at the molecular level. However, by the time the library was ready for the enrichment, *Rpi-Ph3* was published by another team (Zhang et al., 2014). The new probe library was therefore used on a potato population from collaborators (Huazhong Agricultural University, China), B3C1HP, which segregates for *P. infestans* resistance. The bait library was, however, assessed *in silico*, to establish if it would have successfully enriched for the novel *Rpi-Ph3* gene and paralogous sequences.

I. Introduction

Recent advances in genome sequencing technologies enable rapid analysis of entire crop genomes and have accelerated the identification of functional *R* genes. Indeed, eleven years since sequencing the model plant *Arabidopsis thaliana*, the genomes of two important *Solanaceae* crop plants, potato and tomato, were reported (PGSC, 2011; TGC, 2012). These genomes provide a blueprint for the identification of genes coding for important traits such as disease resistance. In the sequenced S*olanum tuberosum* group Phureja clone DM1-3 516

R44 (DM), 755 NB-LRR genes have been identified and their phylogenetic relationships as well as their physical locations in the 12 potato chromosomes described (Jupe et al., 2012; 2013). Similar work was performed on the *S. lycopersicum* linage Heinz 1706 and 397 NB-LRR genes were annotated on the 12 tomato chromosomes (Andolfo et al. 2014). These studies formed the basis of a novel *R* gene enrichment and sequencing platform (RenSeq) that enables the improved annotation of resistance genes in sequenced genomes and facilitates rapid mapping and cloning of resistances via bulked-segregant analysis (Jupe et al., 2013).

In tomato, effective resistances to *P. infestans* are limited, and breeders are mainly relying on *Rpi-Ph2* and/or *Rpi-Ph3* genes, which have been introgressed into several commercial lines (Foolad et al., 2008). These genes reside on chromosome 10 and 9, respectively (Moreau et al., 1998, Chunwongse et al., 2002, Zhang et al., 2013) and provide resistance towards many US isolates of *P. infestans*. Initially, little was known about these two genes when my PhD study commenced. However, significant advances were made on *Rpi-Ph3* with its fine mapping being reported in 2013 (Zhang et al., 2013) and the gene was then cloned a year later (Zhang et al., 2014).

Objective of this chapter:

The primary aim of my study was to create a new probe library for NB-LRRs to realise a RenSeq analysis on *Rpi-Ph3* populations. This new library was designed to take into account the 331 additional *R* genes annotated in the potato genome by Jupe et al., (2013) and to include the improved NB-LRRs annotation of tomato (Andolfo et al., 2014). An *in silico* analysis of the *Rpi-Ph2* and *Rpi-Ph3* loci was performed, to test the hypothesis that NB-LRRs could underpin the resistance phenotype prior to committing to a Renseq approach. Unfortunately, by the time the new probe library was ready, another team published the isolation of *Rpi-Ph3* (Zhang et al., 2014), and changed the focus of my work. Instead of proceeding to the enrichment of the *Rpi-Ph3* segregating population, an *in silico* analysis was

performed to assess if the new probe library would have enabled the identification of *Rpi-Ph3* and homologues sequences. The sequence of *Rpi-Ph3* was also used to design *Rpi-Ph3*-specific PCR markers as a tool for breeders. The new probe library was used on the diploid potato population B3C1HP instead, that segregates 1:1 for late blight resistance. This work was done in collaboration with a group from Huazhong Agricultural University, Wuhan China where the phenotyping and DNA extractions took place.

II. Results

1. Design of a new probe library based on all the predicted *Solanum* NB-LRRs to date

The new bait library was designed to include the 755 predicted NB-LRRs of potato (Jupe et al., 2013), the 397 tomato (Andolfo et al., 2014) and a list of 53 known *R* genes and *R* gene analogs (RGAs) (Jupe et al., 2013). The design of a new probe library facilitated a 2x tiling of all genes by overlapping the 120bp long baits by 60 nucleotides (nt) and was initially computed with the help of the Agilent SureDesign team (Agilent Technologies). I then conducted further tests on the 47,636 probe sequences (Figure III.1-A). A check for sequence redundancy removed 600 identical probes and the remaining 47,036 unique probes were run through RepeatMasker (http://www.repeatmasker.org/, Institute for Systems Biology). This analysis identified 800 probes that represent repetitive regions and were subsequently deleted from the library design. The remaining 46,236 probes were further analysed for specificity and redundancy by establishing their potential targets within the genome at 80% mapping stringency. The 80% mapping stringency had been experimentally established and represents the minimum amount of sequence homology required to facilitate on-target capture under stringent hybridisation conditions (Jupe et al., 2013). Only 20 probes hit more than 80 genes within the potato genome (Figure III.1-A). Those probes were then BLASTed against nr and four of them hit a resistance gene. Those four probes were kept in the list

while the 16 others were deleted (Figure III.1-B). The remaining 46,220 probes were tested for coverage and all the NB-LRRs were covered by between 2 and 951 probes (Figure III.1-C).

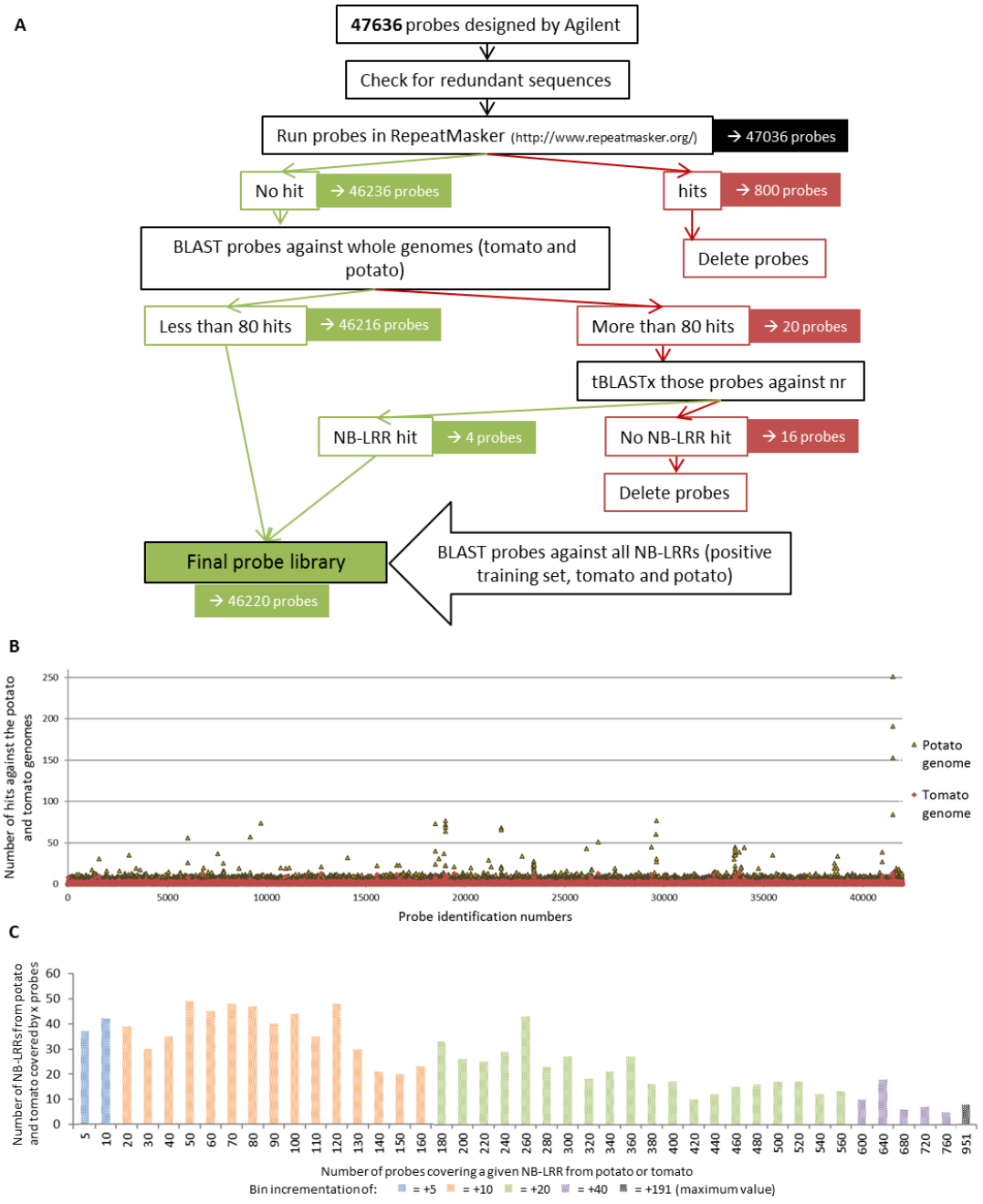

Figure III.1: Design of the new probe library for potato and tomato NB-LRR gene enrichment. A) Schematic of the pipeline used to design the new probe library, including the number of probes deleted (in red) and retained (in green) after each step of the analysis. B) Graphical representation of the standard nucleotide BLAST (BLASTn) results of the probes against the potato and tomato genomes. The number of hits for

each probe against the potato genome (Jupe et al., 2013) is represented in brown and the number of hits against the tomato genome (Andolfo et al., 2014) is represented in red. C) Graphical representation of the number of probes covering the 755 NB-LRRs from potato (Jupe et al., 2013), 397 NB-LRRs from tomato (Andolfo et al., 2014) and the set of 53 known R genes (Jupe et al., 2013)

2. Investigating the *Rpi-Ph2* and *Rpi-Ph3* loci

A. *In silico* analysis of the *Rpi-Ph2* and *Rpi-Ph3* loci

a. *Rpi-Ph2* maps to the potato *Rpi-ber2* locus

At the time of the study, the only genetic information available for *Rpi-Ph2* were two markers, CP105 and TG233 on chromosome 10 of tomato, that were associated with the resistance (Moreau et al., 1998). A collaborator in the US (John W. Scott, University of Florida) has developed additional markers for *Rpi-Ph2* tomato breeding. These markers are located near position 64Mb on chromosome 10. Using these data and the annotated genome of tomato (Tomato genome sequencing consortium, 2012; Andolfo et al., 2013; Jupe et al., 2013; Andolfo et al., 2014), three NB-LRR genes (Solyc10g085460.1.1, Solyc10g085780.1.1 and Solyc10g086590.1.1) have been identified in the corresponding, non-functional *Rpi-Ph2* region of the sequenced tomato line Heinz 1706. Those genes have been BLASTed (BLASTn) against the annotated DM NB-LRRs (Jupe et al., 2012; Jupe et al., 2013; Andolfo et al., 2014) and interestingly, Solyc10g085460 shows strong similarities to the potato late blight resistance gene *Rpi-ber2* on chromosome 10 (Jupe et al., 2013) (Figure III.2-A).

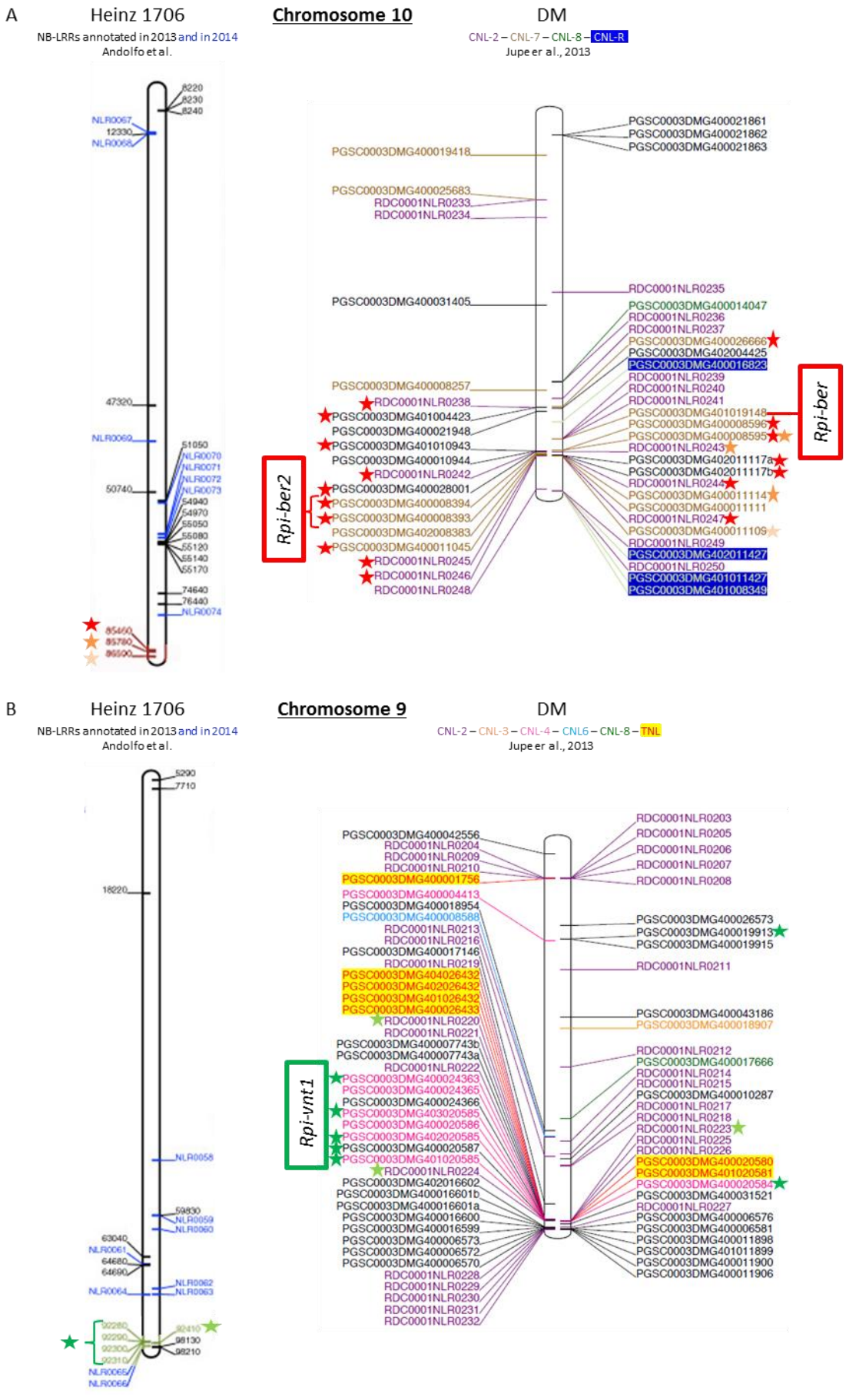
A
Heinz 1706
NB-LRRs annotated in 2013 and in 2014
Andolfo et al.
Chromosome 10
DM
CNL-2 – CNL-7 – CNL-8 – CNL-R
Jupe er al., 2013
8220
8230
8240
NLR0067
12330
NLR0068
47320
NLR0069
51050
NLR0070
NLR0071
NLR0072
NLR0073
50740
54940
54970
55050
55080
55120
55140
55170
74640
76440
NLR0074
85460
85780
86500
PGSC0003DMG400019418
PGSC0003DMG400025683
RDC0001NLR0233
RDC0001NLR0234
PGSC0003DMG400031405
PGSC0003DMG400008257
RDC0001NLR0238
PGSC0003DMG401004423
PGSC0003DMG400021948
PGSC0003DMG401010943
PGSC0003DMG400010944
RDC0001NLR0242
PGSC0003DMG400028001
PGSC0003DMG400008394
PGSC0003DMG400008393
PGSC0003DMG402008383
PGSC0003DMG400011045
RDC0001NLR0245
RDC0001NLR0246
RDC0001NLR0248
Rpi-ber2
PGSC0003DMG400021861
PGSC0003DMG400021862
PGSC0003DMG400021863
RDC0001NLR0235
PGSC0003DMG400014047
RDC0001NLR0236
RDC0001NLR0237
PGSC0003DMG400026666
PGSC0003DMG402004425
PGSC0003DMG400016823
RDC0001NLR0239
RDC0001NLR0240
RDC0001NLR0241
PGSC0003DMG401019148
PGSC0003DMG400008596
PGSC0003DMG400008595
RDC0001NLR0243
PGSC0003DMG402011117a
PGSC0003DMG402011117b
RDC0001NLR0244
PGSC0003DMG400011114
PGSC0003DMG400011111
RDC0001NLR0247
PGSC0003DMG400011109
RDC0001NLR0249
PGSC0003DMG402011427
RDC0001NLR0250
PGSC0003DMG401011427
PGSC0003DMG401008349
Rpi-ber
B
Heinz 1706
NB-LRRs annotated in 2013 and in 2014
Andolfo et al.
Chromosome 9
DM
CNL-2 – CNL-3 – CNL-4 – CNL6 – CNL-8 – TNL
Jupe er al., 2013
5290
7710
18220
NLR0058
59830
NLR0059
NLR0060
63040
NLR0061
64680
64690
NLR0062
NLR0063
NLR0064
92280
92290
92300
92310
92410
98130
98210
NLR0065
NLR0066
PGSC0003DMG400042556
RDC0001NLR0204
RDC0001NLR0209
RDC0001NLR0210
PGSC0003DMG400001756
PGSC0003DMG400004413
PGSC0003DMG400018954
PGSC0003DMG400008588
RDC0001NLR0213
RDC0001NLR0216
PGSC0003DMG400017146
RDC0001NLR0219
PGSC0003DMG404026432
PGSC0003DMG402026432
PGSC0003DMG401026432
PGSC0003DMG400026433
RDC0001NLR0220
RDC0001NLR0221
PGSC0003DMG400007743b
PGSC0003DMG400007743a
RDC0001NLR0222
PGSC0003DMG400024363
PGSC0003DMG400024365
PGSC0003DMG400024366
PGSC0003DMG403020585
PGSC0003DMG400020586
PGSC0003DMG402020585
PGSC0003DMG400020587
PGSC0003DMG401020585
RDC0001NLR0224
PGSC0003DMG402016602
PGSC0003DMG400016601b
PGSC0003DMG400016601a
PGSC0003DMG400016600
PGSC0003DMG400016599
PGSC0003DMG400006573
PGSC0003DMG400006572
PGSC0003DMG400006570
RDC0001NLR0228
RDC0001NLR0229
RDC0001NLR0230
RDC0001NLR0231
RDC0001NLR0232
Rpi-vnt1
RDC0001NLR0203
RDC0001NLR0205
RDC0001NLR0206
RDC0001NLR0207
RDC0001NLR0208
PGSC0003DMG400026573
PGSC0003DMG400019913
PGSC0003DMG400019915
RDC0001NLR0211
PGSC0003DMG400043186
PGSC0003DMG400018907
RDC0001NLR0212
PGSC0003DMG400017666
RDC0001NLR0214
RDC0001NLR0215
PGSC0003DMG400010287
RDC0001NLR0217
RDC0001NLR0218
RDC0001NLR0223
RDC0001NLR0225
RDC0001NLR0226
PGSC0003DMG400020580
PGSC0003DMG401020581
PGSC0003DMG400020584
PGSC0003DMG400031521
RDC0001NLR0227
PGSC0003DMG400006576
PGSC0003DMG400006581
PGSC0003DMG400011898
PGSC0003DMG401011899
PGSC0003DMG400011900
PGSC0003DMG400011906

Figure III.2: Schematic representation of A) the *Rpi-Ph2* (in red) and B) the *Rpi-Ph3* (in green) regions on the tomato (Heinz 1706) chromosomes (left) 10 and 9, respectively. The homologues genes in potato (DM) are shown on the right with colour coded stars. The cloned potato *Rpi* genes in the regions of interest are annotated. Adapted from Jupe et al., 2013 and Andolfo et al., 2014.

b. *Rpi-Ph3* map to the potato *Rpi-vnt1* locus

Similar to that for *Rph-Ph2*, there was little genetic knowledge on *Rpi-Ph3* at the time of this study. Breeders have been using *Rpi-Ph3* associated markers on chromosome 9, such as TG591A and L87RF (Chunwongse et al., 2002). Markers closer to the resistance have been developed around position 66-67 Mb by John W Scott (personal communication). The same analysis as described above was conducted on the annotated chromosome 9 of tomato (Andolfo et al., 2013; Andolfo et al., 2014) and five NB-LRR genes have been identified in the *Rpi-Ph3* region (Solyc09g092280.1.1, Solyc09g092290.1.1, Solyc09g092300.2.1, Solyc09g092310.1.1 and Solyc09g092410.2.1,) (Figure III.2 B), of which the first four are part of the *Tm*^{2} cluster (Jupe et al., 2012). This cluster corresponds to the *Rpi-vnt1* cluster in the potato genome, which is another characterised *R* gene against *P. infestans* (Pel et al., 2009; Foster et al., 2009). Indeed, when BLASTed (BLASTn) against the potato NB-LRR, those five tomato genes gave hits within the *Rpi-vnt1* cluster (Figure III.2 B).

B. *Rpi-vnt1* PCR screen shows that *Rpi-Ph3* is different from *Rpi-vnt1*

The primer pairs Rpi-vnt1_Full (which amplifies full-length Rpi-vnt1) and Rpi-vnt1_diag (which amplifies a region conserved in *Rpi-vnt1* and closely related homologs) were successfully tested on a DNA sample extracted from a *Rpi-vnt1* transgenic potato *cv.* Désirée, in parallel to water as a negative control. An association panel of tomato lines either containing *Rpi-Ph2*, *Rpi-Ph3* or neither of these two genes was tested by PCR for the presence/absence of *Rpi-vnt1*. DNA from *Rpi-vnt1* transgenic potato cv. Désirée and no-template control (called water) were used as a positive and negative control, respectively

(Figure III.3). The diagnostic primers (Rpi-vnt1_diag_F_257 and Rpi-vnt1_diag_R_85) amplified fragments of the expected 446bp size in all the tomato lines, showing that genes related to the *Rpi-vnt1* family were present in those lines. However, the full length Rpi-vnt1 primers (Rpi-vnt1_Full_F_130 and Rpi-vnt1_Full_R_135) specific to the functional *Rpi-vnt1* gene did not amplify the expected 2676bp (*Rpi-vnt1.1* allele) or 2718bp (*Rpi-vnt1.2* and *Rpi-vnt1.3* alleles) fragments in any of the lines, showing that the tomato association panel used in this study carries no functional *Rpi-vnt1* gene.

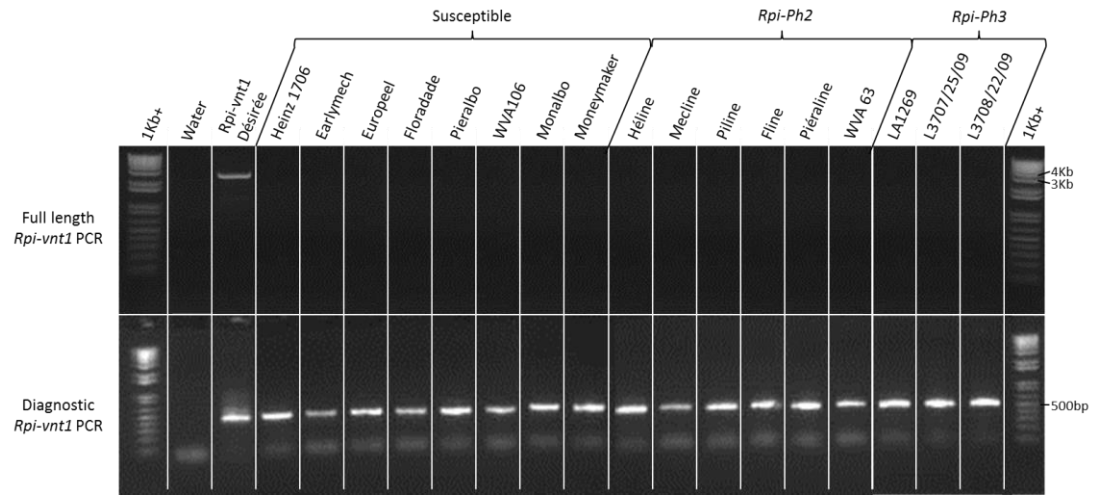

Figure III.3: PCR results of 17 tomato lines from the association panel. DNA templates included the positive control extracted from the transgenic potato *cv.* Désirée expressing *Rpi-vnt1*, water as a negative control, eight susceptible lines without *Rpi-Ph2* or *Rpi-Ph3*, seven *Rpi-Ph2* containing lines and three *Rpi-Ph3* containing lines. PCRs were performed with the Full length (top panel) and diagnostic (bottom panel) *Rpi-vnt1* primers. Pictures were taken under UV light, after electrophoreses on a 1% agarose gel supplemented with 0.5µg/mL ethidium bromide. The ladder 1Kb Plus from Invitrogen was used in each gel.

C. Design of *Rpi-Ph3* specific markers

After the isolation of *Rpi-Ph3* was published (Zhang et al, 2014), two sets of primers were designed that facilitated specific amplification of *Rpi-Ph3* compared to the sequences

available in the tomato and potato genomes alongside reported *Rpi-Ph3* homologous genes. The primers were designed to enable breeders and collaborators/funders in the US to implement marker specific selection for *Rpi-Ph3* resistance in cultivar selection. The first step was to BLAST the *Rpi-Ph3* sequence (Yuling Bai personal communication) against the tomato genome (Jupe et al., 2013, Andolfo et al., 2014), to identify closely related but non-functional homologs in the susceptible tomato line Heinz 1706. The tomato annotated *R* genes SL2.40ch09 number 018220, 092280, 092290, 092300 and 092310 had more than 80% identity to *Rpi-Ph3*. As expected, the cloned potato genes *Rpi-vnt1.1* from *S. venturii* (Foster et al., 2009, Pel et al., 2009) and *Rpi-mcq1* from *S. mochiquense* (Jones et al., 2009), and the tobacco Tm-2^2 (Lanfermeijer et al., 2003), share a high level of sequence identity with *Rpi-Ph3* (Zhang et al., 2014). In the Table III.1, the percentage of identity of the different sequences described before are defined.

Table III.1: Similarity matrix of *Rpi-Ph3* and homologs. Displayed are the potato *Rpi-Mcq1.1*, *Rpi-Mcq1.2*, *Rpi-vnt1.1*, *Rpi-vnt1.2*, *Rpi-vnt1.3* and *R9a*; the tomato annotated *R* genes SL2.40ch09 number 018220, 092280, 092290, 092300 and 092310; and the cloned tomato *R* gene Tm-2^2. The percentage of identity is colour-coded in grey, with the darker grey highlight showing the higher percentage of identity. The matrix was built using Geneious 6.1.8.

Nt sequences	*Rpi-Ph3*	*Rpi-mcq1.1*	*Rpi-mcq1.2*	*Rpi-vnt1.1*	*Rpi-vnt1.2*	*Rpi-vnt1.3*	*R9a*	*SL2.40ch09 018220*	*SL2.40ch09 092280*	*SL2.40ch09 092290*	*SL2.40ch09 092300*	*SL2.40ch09 092310*	*Tm-2^2*
Rpi-Ph3		85.1	84.7	84.8	84.8	84.8	83.3	82.1	86.8	87.6	94.3	95.4	81.9
Rpi-mcq1.1	85.1		87.9	87.7	87.6	87.6	87.6	85.2	80.2	80.9	85.4	85.7	85.3
Rpi-mcq1.2	84.7	87.9		87.4	87.4	87.4	85.1	84	79.7	79.9	84.8	85.5	84.2
Rpi-vnt1.1	84.8	87.7	87.4		98.2	98.2	85.6	84.2	79.1	79.3	85	84.9	84.1
Rpi-vnt1.2	84.8	87.6	87.4	98.2		100	85.6	84.3	79.1	79.4	85	84.9	84.2
Rpi-vnt1.3	84.8	87.6	87.4	98.2	100		85.6	84.3	79.1	79.4	85	84.9	84.2
R9a	83.3	87.6	85.1	85.6	85.6	85.6		82.9	79.3	80.2	83.4	83.7	82.9
SL2.40ch09_018220	82.1	85.2	84	84.2	84.3	84.3	82.9		77.3	77.8	82	82.4	97.5
SL2.40ch09_ 092280	86.8	80.2	79.7	79.1	79.1	79.1	79.3	77.3		94.8	85.2	86.9	77.3
SL2.40ch09_092290	87.6	80.9	79.9	79.3	79.4	79.4	80.2	77.8	94.8		86	87.4	77.9
SL2.40ch09_092300	94.3	85.4	84.8	85	85	85	83.4	82	85.2	86		96.4	81.7
SL2.40ch09_092310	95.4	85.7	85.5	84.9	84.9	84.9	83.7	82.4	86.9	87.4	96.4		82.1
Tm-2^2	81.9	85.3	84.2	84.1	84.2	84.2	82.9	97.5	77.3	77.9	81.7	82.1	

All these homologous sequences were taken into account for the design of *Rpi-Ph3* specific primers. The translated amino acid sequences of the corresponding genes were aligned to

Rpi-Ph3 to help selecting the most distinct regions for the primer design. Regions specific to *Rpi-Ph3* were identified (Figure III.4-A) and used to design a set of primers amplifying the full length *Rpi-Ph3* (Rpi-Ph3_Full primers), and another set amplifying a shorter portion of *Rpi-Ph3* (Rpi-Ph3_diag primers) (Table II.5, Figure III.4-B).

Figure III.4: Alignments of *Rpi-Ph3* and closely related genes. A) Amino acid alignment of (**1**) the tomato *Rpi-Ph3*; (**2**) the potato *Rpi-Mcq1.1*, (**3**) *Rpi-Mcq1.2*, (**4**) *Rpi-vnt1.1*,

(**5**) *Rpi-vnt1.2*, (**6**) *Rpi-vnt1.3*; the tomato annotated *R* genes SL2.40ch09 number (**7**) 018220, (**8**) 092280, (**9**) 092290, (**10**) 092300, (**11**) 092310 and (**12**) the cloned tomato *R* gene Tm-2². (**A**) The consensus sequence is shown above the alignment. The primers are represented in dark green (forward) and light green (reverse), above the *Rpi-Ph3* sequence. Yellow labels above the *Rpi-Ph3* sequence point to the amino acids that are unique to *Rpi-Ph3*. B) Equivalent nucleotide alignment of the corresponding primer binding sites. *Rpi-Ph3* (=1) is set as the reference and only varying nucleotide in the rest of the sequences are highlighted in colours. The alignments were performed in Geneious 6.1.8.

When tested, the Rpi-Ph3_Full set of primers amplified the full-length *Rpi-Ph3* gene and yielded the expected 2556bp product in all of the five *Rpi-Ph3* tomato lines. Indeed, compared to the *Rpi-vnt1* analysis conducted above (Figure III.3), the number of *Rpi-Ph3* containing lines was expanded from three to five, to improve the robustness and confidence associated with the PCR analysis. In contrast to *Rpi-Ph3* containing lines, no amplification product was observed in the remaining tomato lines (susceptible and *Rpi-Ph2*), the water control, and DNA samples from *N. benthamiana*, the transgenic *Rpi-vnt1* potato *cv.* Désirée, *Nicotiana sylvestris* and two varieties of pepper (California wonder and CM334) (Figure III.5). Similarly, the Rpi-Ph3_diag set of primers amplified a unique portion of *Rpi-Ph3*, with the expected size of 293bp in only the *Rpi-Ph3* tomato lines tested (Figure III.5). The DNA sample quality was tested with a set of universal 18S primers, and all of them produced the expected ~100bp amplification product after PCR (Figure III.5).

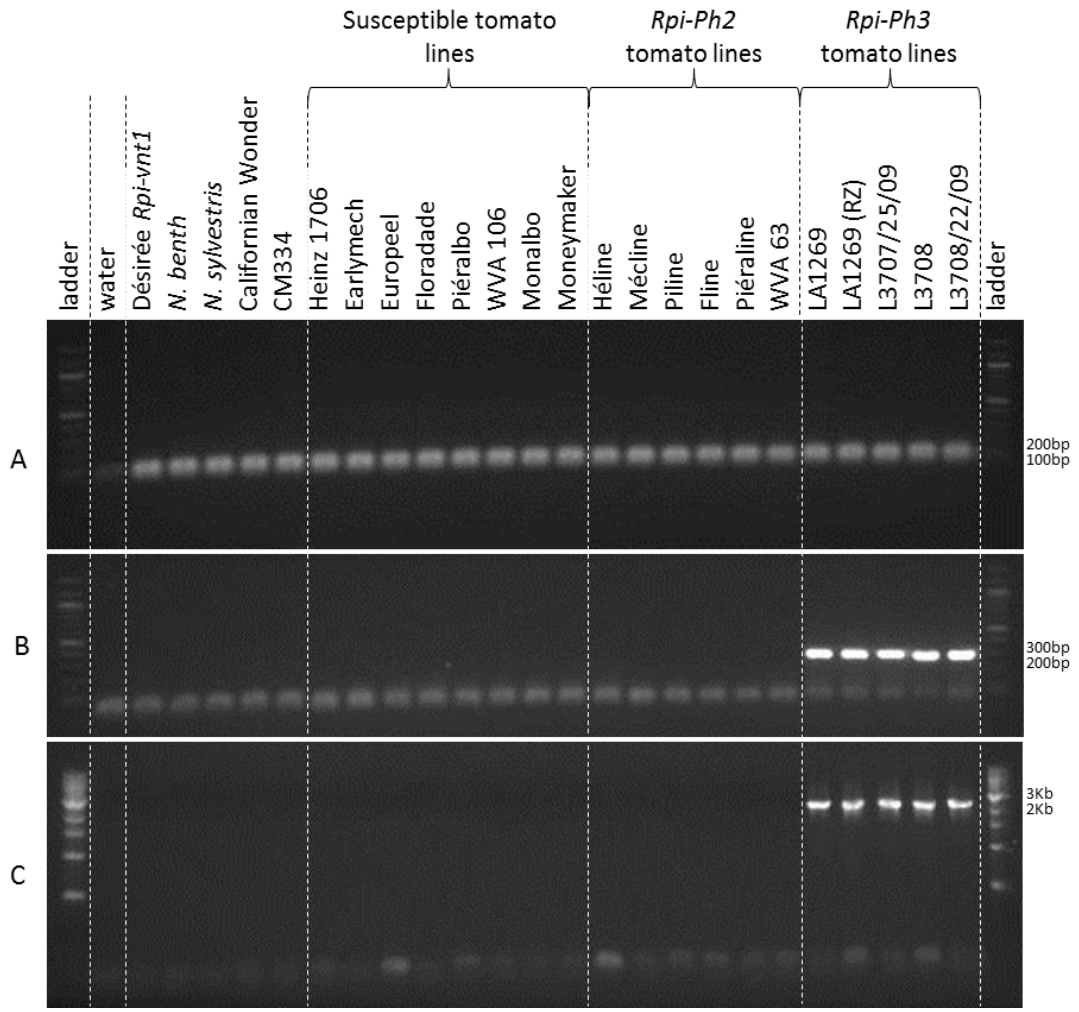

Figure III.5: *Rpi-Ph3* PCR screen of the 19 tomato lines, with the control samples potato Désirée *Rpi-vnt1*, *N. benthamiana*, *N. sylvestris*, the peppers Californian Wonder and CM334 and the control water. PCR were performed with **A:** the 18S primers, **B:** the Rpi-Ph3_diag primers and **C:** the Rpi-Ph3_Full primers. Pictures were taken under UV light, after electrophoresis in a 1% agarose gel supplemented with 0.5µg/mL ethidium bromide. **A** and **C** were loaded with the quick-load 100bp ladder from NEB and **B** was loaded with the quick-load 1Kb ladder from NEB. Sizes of the interesting ladder bands are specified.

D. Evaluating the suitability of RenSeq library for the identification of *Rpi-Ph3*

As mentioned previously, the *Rpi-Ph3* gene was cloned by Zhang et al., (2014) as the new NB-LRR gene bait library was designed. To ascertain if the library would have been suitable to

identify *Rpi-Ph3* as a candidate (e.g. facilitate enrichment of this particular gene) an *in silico* analysis was conducted. The probe library was BLASTed against the cloned *Rpi-Ph3* sequence (Yuling Bai personal communication) and closely related genes, including unpublished *R9a* (Jack Vossen personal communication), *Rpi-mcq1.1* (Jones et al., 2009), *Rpi-vnt1.1* (Foster et al., 2009) and Tm-2² (Lanfermeijer et al., 2003). Of the 46220 probes within the new library, 306 probes targeted *Rpi-Ph3*, 276 probes *R9a,* 334 probes *Rpi-mcq1.1*, 331 *Rpi-vnt1.1* and 248 mapped to *Tm-2²* with more 80% identity over an alignment length of more than 115bp. Figure III.6 presents the blast outcome in the format of a graphical heat map. The analysis further revealed that 47 probes had sufficient homology to all five resistance genes with more than 80% identity and an alignment length of more than 115bp. This figure was created using the application BLASTmap developed by Dr. Katie Baker (https://kbio.shinyapps.io/BLASTmap/). In addition, 273 probes with over 95% homology and an alignment length of more than 115bp were identified for *R9a*, 285 for *Rpi-Ph3*, 233 for *Tm-2²*, 330 for *Rpi-mcq1.1* and 313 for *Rpi-vnt1.1*.

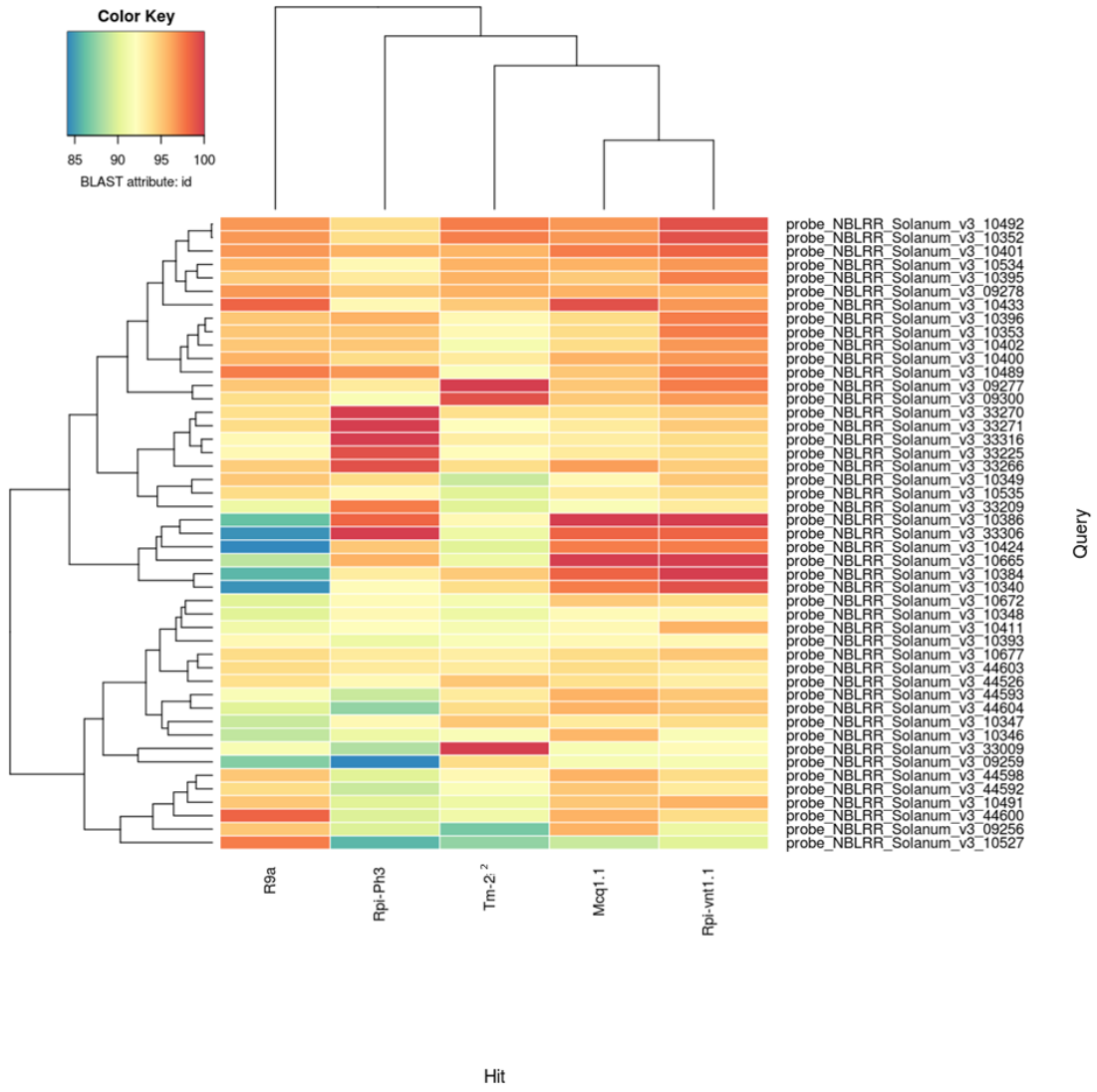

Figure III.6: Graphical representation of the probe library BLAST against *Rpi-Ph3* and closely related resistance genes *R9a*, *Tm2²*, *Mcq1.1* and *Rpi-vnt1.1*. Shown are the 47 probes covering all five R genes with more than 80% identity over more than 115 nucleotides coverage length. This figure was created using the application BLASTmap developed by Dr. Katie Baker (https://kbio.shinyapps.io/BLASTmap/).

3. Using RenSeq to identify new *Rpi* genes

A. B3C1HP population enrichment

With the help of Dr. Miles Armstrong, the new probe library was used to help fine map a single late blight resistance gene that is segregating in the diploid potato population B3C1HP (Li et al., 2015). The expanded population was screened for recombinants and phenotyped

by our collaborators in China (Prof Xie Conghua, Prof Tian Zhendong and Jiang Rui from Huazhong Agricultural University, Wuhan China). DNA samples were obtained from the heterozygous resistant parent (R/S), the homozygous susceptible parent (S/S), a bulk of 27 heterozygous resistant progeny (BR) and a bulk of 27 homozygous susceptible progeny (BS). Prior to indexing and enrichment, three different fragmentation conditions (TableII.8) were tested on the bulk susceptible and the bulk resistant to obtain approximately 500bp long inserts. The results from the Bioanalyser showed that condition #2; consisting of a 50W peak power, a 20%, duty factor, 200 cycles per burst and 60s treatment time; was the most suitable. It resulted in an average fragment size of 537bp for the bulk susceptible and 603bp for the bulk resistant (Table III.2). Indeed, the optimal fragment size for Illumina paired-end sequencing is 500bp as this allows contiging of 2x300bp paired-end reads prior to read mapping.

Table III.2: Bioanalyser results of the 3 different conditions of fragmentation tested on the bulks susceptible and resistant.

Average size (bp) for	Conditions #1	Conditions #2	Conditions #3
Bulk susceptible	740	537	986
Bulk resistant	745	603	969

The susceptible and resistant parents were fragmented under condition #2. The four samples were then ligated with Illumina adaptors and PCR amplified with eight cycles to apply the barcodes to the adaptors. The quality of the samples was checked again through the Bioanalyser and sizes peaked at the expected ~500bp length (Figure III.7). The capture was then performed at 65°C for 24 hours followed by 10 cycles of PCR according to Van Weymers et al., (2016). The PCR step generated a total of 229.69ng of DNA, which was sent for paired-end MiSeq sequencing using 2x300 chemistry.

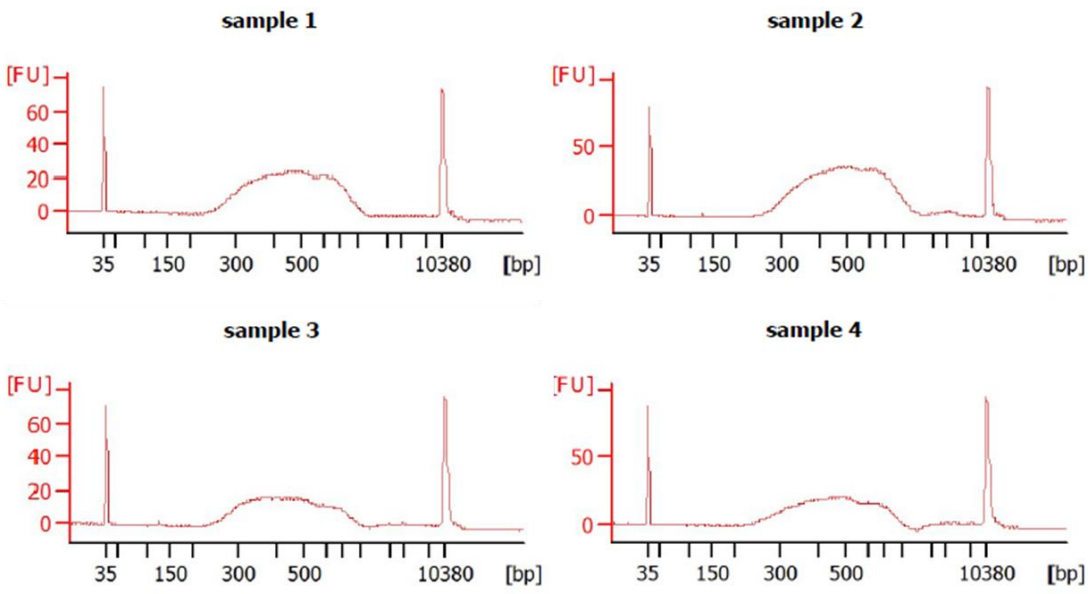

Figure III.7: Bioanalyser size distribution of the DNA samples after fragmentation and adapter ligation. Sample 1: resistant parent, sample 2: susceptible parent, sample 3: bulked resistant sample and sample 4: bulked susceptible sample of the B3C1HP population.

B. B3C1HP population RenSeq analysis.

After enrichment of the different samples, 1,286,701 high quality paired-ends reads were obtained for the parent resistant, 1,582,117 for the parent susceptible, 1,866,832 for the bulk resistant, and 1,899,506 for the bulk susceptible. These were analysed for informative single nucleotide polymorphisms (SNPs), as detailed in Figure III.8. In essence, SNPs were identified between bulked resistant and bulked susceptible samples that corresponded to the expected allele frequency in a diploid cross. Using DM as a reference, BS and BR reads were individually mapped at 1, 2, 5 and 10% mismatch rates. SNPs that displayed no alternative allele in the BS (+/- 10%) but an alternative allele frequency of 50% (+/- 10%) in R bulk were selected as informative. These SNPs were compared to the polymorphism identified between the heterozygous resistant parent and the homozygous susceptible parents. Only SNPs that could be corroborated in the bulks and parents were retained. Lastly, as more polymorphic genes could skew the analysis, SNPs were placed back on individual

genes and only the numbers of genes with informative SNPs are displayed. The computational RenSeq analysis and SNP calling was performed by Dr. Katie Baker and a clear pick of informative SNPs was visible at the end of chromosome IX (Figure III.9).

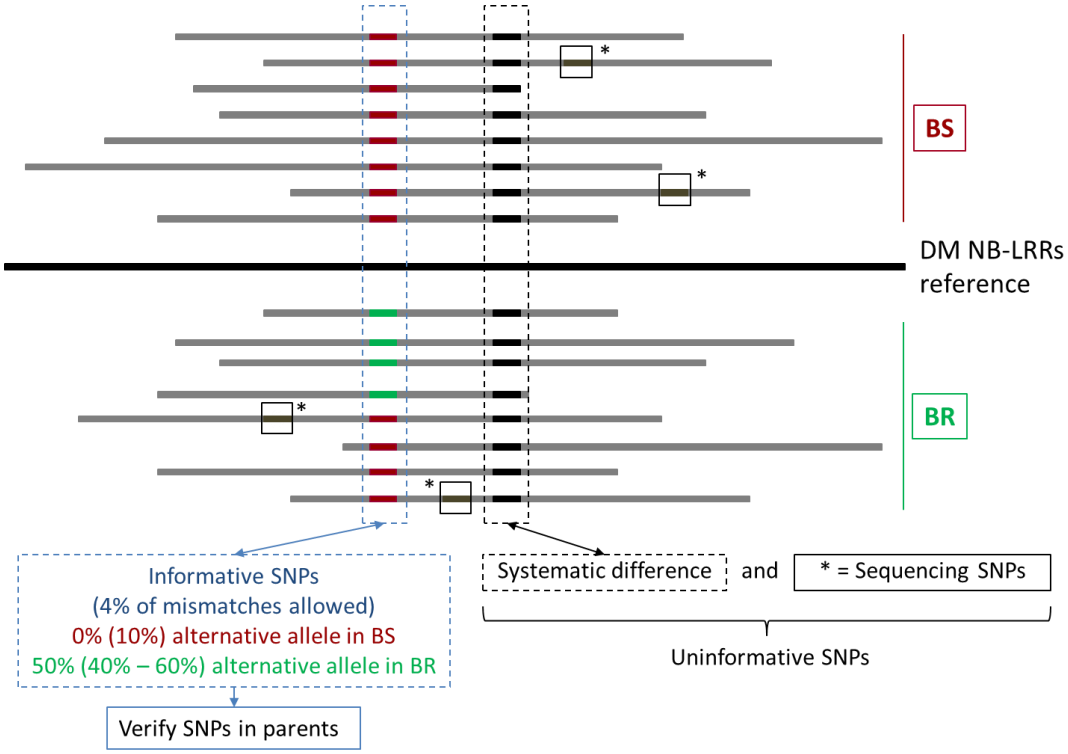

Figure III.8: Schematic of the Single Nucleotide Polymorphism (SNP) analysis involving bulked susceptible (BS), bulked resistant (BR) and parental Illumina MiSeq reads following RenSeq analysis. Sequencing errors (outlined in black with an asterix), and systemic differences (outlined in dotted black) between the reference genome and the target samples are dismissed. Informative SNPs that remain after the bulking strategy and that conform to the expect alternative allele frequency (outlined in dotted blue) are retained if they are also identified in the comparison between the parents.

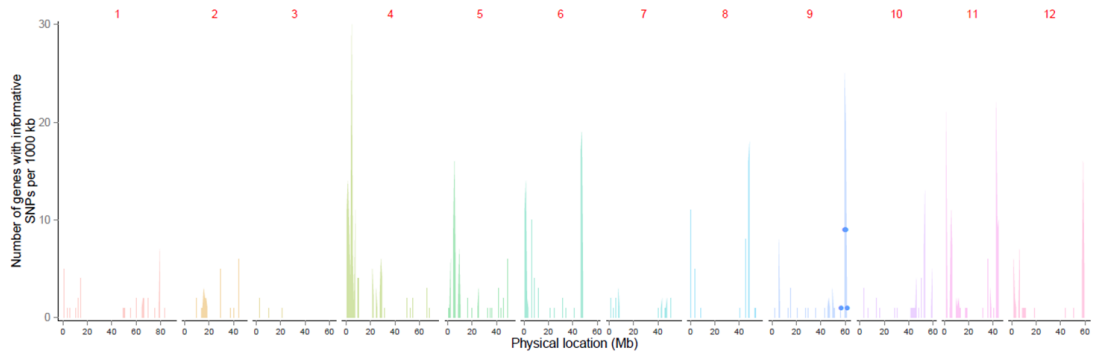

Figure III.9: Graphical representation of the informative SNPs found after RenSeq analysis of the BC31HP population at 5% mismatch rate, across the whole potato genome. This figure was generated by Dr. Katie Baker (JHI). Each chromosome is represented in a different colour and labelled with its number. The smears represent the annotated genes on the different chromosomes. The dots represent the informative SNPs, which are only present in chromosome 9 (in blue).

III. Discussion

The *in silico* analysis of the tomato *Rpi-Ph2* locus revealed that it is equivalent to the potato locus carrying the potato resistance genes *Rpi-ber* (Rauscher et al., 2006; Park et al, 2009) and *Rpi-ber2* (Jupe et al., 2013) on chromosome 10 (Figure III.2A). Similarly, the *Rpi-Ph3* locus corresponds to the potato *Rpi-vnt1* locus on chromosome 9 (Pel et al., 2009, Foster et al., 2009) (Figure III.2B). This finding was corroborated by the fine mapping and the eventual cloning of the *Rpi-Ph3* gene by Zhang et al., (2013; 2014). It also confirmed the high level of sequence identity shared with the potato genes *Rpi-vnt1.1* from *S. venturii* (Pel et al., 2009, Foster et al., 2009), *Rpi-mcq1* (Jones at al., 2009) and *R9a* (Jo et al., 2015). It is interesting to note that both *Solanaceae* plants, tomato and potato, have similar 'hot spots' of resistance genes that, in the cases of *Rpi-Ph2* and *Rpi-Ph3*, provide resistances to the late blight pathogen *P. infestans*. However, in the case of *Rph-Ph3*, the similarity to the tomato mosaic virus resistance gene *Tm-2²* (Lanfermeijer et al., 2003) also suggests that other pathogen resistances have arisen in this locus. Compared to potato, tomato appears to have fewer NB-

LRR genes (Andolfo et al., 2014), albeit common ancestral origins are evident based on sequence homology. This, for example, could be a consequence of different pathogen pressures and/or selection. It also remains to be seen if the functional resistances with high level of sequence identity in both *Solanum* species are based on similar recognition events and/or host response mechanism. Pathogen effectors provide a tool that allows this question to be addressed (Vleeshouwers et al., 2008 and 2011; Vleeshouwers and Oliver; 2014) and in Chapter 5, the case of *Rpi-Ph3*and its homology to *Rpi-vnt1* is assessed.

Despite the fact that *Rpi-Ph3* was cloned during the lifetime of this project, which required me to shift the focus of my study, we successfully collaborated with the group and designed two sets of primers specific to *Rpi-Ph3* (Figure III.5). These primers were shared with a tomato breeder in the US (Dr. John Scott, University of Florida) to provide a tool for marker-assisted selection (MAS) in tomato breeding programs. Similarly, the cloning of the *Rpi-Ph3* gene, for which no bespoke baits were designed, allowed me to test the efficacy of the new bait library *in silico*. Figure III.6 indicates that *Rpi-Ph3* and its related genes would have been pulled out of the enrichment process, if we assume that 95% sequence identity between probes and targets is sufficient. Indeed, some probes displayed 100% sequence identity to *Rpi-Ph3*, which suggest that the corresponding parts of the functional gene are highly conserved amongst genes in reference genomes of DM or Heinz.

After confirming that the new bait library is inclusive enough to facilitate the enrichment of sequence related but novel genes, the RenSeq strategy was applied to aid the fine mapping of the late blight resistance in the potato population B3C1HP (Li et al., 2015). In line with the SSR marker analysis conducted by Li et al., (2015), the RenSeq analysis showed that the resistance was located on the lower end of chromosome IX (Figure III.8). Coincidently, this region also corresponds to the location of *Rpi-Ph3*, *Rpi-vnt1* (Foster et al., 2009, Pel et al.,

2009), *Rpi-mcq1* (Smilde et al., 2005) and *R9a* (Jo et al., 2015) and provides further evidence that multiple resistances originate in this locus.

Although not shown in this chapter, the RenSeq analysis was expanded to a novel PVY resistance that originate from the wild species *Solanum chacoense* and the analysis mapped the resistance also to this locus on the long arm of chromosome 9. It therefore remains to be seen if there is sequence homology between the PVY resistance and the Tm-2 resistance. However, I have shown that the new probe library design is applicable to study resistances against other pathogens.

Chapter IV: Utilising 'Omics' technologies to identify and prioritise novel sources of resistance to the oomycete pathogen *Phytophthora infestans* in potato germplasm collections

This chapter details how existing germplasm collections can be screened rapidly and effectively with 'omic' technologies. A combination of next generation sequencing-based 'genomics' in combination with NB-LRR gene target enrichment and 'effectoromics' was utilised to help prioritise resistances. A screen of 126 wild diploid *Solanum* accessions from the Commonwealth Potato Collection (CPC) with *P. infestans* isolates belonging to the genotype 13-A2 has identified resistances in the species *S. bulbocastanum*, *S. capsicibaccatum*, *S. microdontum, S. mochiquense, S. okadae*, *S. pinnatisectum*, *S. polyadenium, S. tarijense* and *S. verrucosum.* A diagnostic adaptation of RenSeq (dRenSeq) has been established and validated as a tool to quickly assess if resistant plants contain already characterised NB-LRR genes. dRenSeq in resistant *S. okadae* accessions 7129, 7625, 3762 and a bulk of 20 resistant progeny confirmed the presence of the full-length *Rpi-vnt1.1* under stringent mapping conditions and corroborated allele mining results in the accessions 7129 and 7625 as well as Avr-vnt1 recognition in transient expression assays. In contrast, the susceptible *S. okadae* accession 3761 and a bulk of 20 susceptible progeny lacked sequence homology in the 5' end compared to the functional *Rpi-vnt1.1* gene. Further evaluation of *S. okadae* accessions with late blight isolates that have a broad spectrum of virulence demonstrated that, although *S. okadae* accessions 7129, 7625 and 7629 contain functional *Rpi-vnt1.1*, they also carry a novel resistance gene.

This work has been published in Frontiers in plant Sciences (Van Weymers et al., 2016) and this chapter recapitulates most of the manuscript. However, in addition, dRenSeq was also applied to the B3C1HP potato population (Li et al., 2015) described in Chapter III and identified R8 as the most likely source of the resistance.

I. Introduction

Potato is the most important non-cereal food crop worldwide and is consumed by more than a billion people (Birch et al., 2012). Pests and pathogens represent a serious and continuing threat to potato production and the most widespread and economically significant of these is late blight, caused by the oomycete pathogen *Phytophthora infestans*.

The ability to withstand multiple biotic and abiotic stresses is critical for wild potato species, suggesting that many unexplored natural sources of resistance exist for exploitation in breeding programs. With the availability of extensive germplasm resources, including the CPC at the James Hutton Institute (Bradshaw et al., 2006), and improved genomics tools, the potential to exploit this natural biodiversity is considerable. Newly identified and deployed resistances could provide an environmentally benign opportunity to secure potatoes as a major food source in the future (Birch et al., 2012). Critical for the success of such disease control is, however, a detailed knowledge of the underlying mechanisms of defence including an understanding of the pathogen molecules recognised to facilitate complementary deployment of resistances.

NB-LRR genes are key to plant immunity and their presence; absence or allelic diversity is decisive for disease resistance. At least seven distinct potato NB-LRRs effective towards *P. infestans* have been cloned so far and their cognate effectors are well described (reviewed in Vleeshouwers and Oliver, 2014). Furthermore, allele mining for late blight resistance genes such as *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* from the diploid Mexican species *S. bulbocastanum* has identified functional orthologs in other species (Lokossou et al., 2009; 2010). For

example, *Rpi-blb1* orthologous genes were identified in the Mexican diploid species *S. cardiophyllum* and the allopolyploid species *S. papita* and *S. polytrichon* (Lokossou et al., 2010). Furthermore, highly conserved homologs of Rpi-blb1 were also identified in *Solanum stoloniferum* and derived crossings (Wang et al 2008). When seeking novel resistances in germplasm collections, it is thus imperative to exclude accessions that contain already characterised resistances as the sole means of defence against the pathogen in question.

Objectives of this chapter:

The objectives of this chapter were to devise an 'omics'-based approach for the screening of the CPC and to prioritise novel resistances toward late blight. The newly developed probe library described in Chapter III was used for the screening of CPC potato accessions and populations with the aim to establish RenSeq as a diagnostic tool (dRenSeq). dRenSeq analysis was applied to CPC accession of *S. okadae* and also to the B3C1HP potato population (Li et al., 2015) detailed in Chapter III.

II. Results

1. Characterisation of the potato material

A. Identification of diploid CPC accessions resistant to *P. infestans* 13-A2 genotype

Seedlings and selected whole plants of 126 diploid CPC accessions belonging to 34 species (Table IV.1) were tested with the late blight isolates 2006-3928A and/or 2009-7654A belonging to the *P. infestans* clonal lineage 13-A2, with the help of Brian Harrower, James Lynott and Gaynor McKenzie (JHI).

Table IV.1: List of diploid CPC accessions tested for late blight resistance.

Species	CPC accession	Species	CPC accession	Species	CPC accession
S. alandiae	7212	*S. commersonii*	5855	*S. palustre*	2451
	7324		5858	*S. pampasense*	7066
S. berthaultii	5701		5861		7068
S. boliviense	7026		7058		7328
	7320		7520		7610
	7335	*S. ehrenbergii*	7507	*S. pinnatisectum*	7661
S. brachistotrichum	3822		7510		2301
S. brevicaule	7704	*S. gourlayi*	7671		3559
	7705		7672		3863
	7709	*S. infundibuliforme*	2477		7521
	7753	*S. kurtzianum*	5864		7659
	7754		5889	*S. polyadenium*	3501
	7755	*S. marinasense*	6020		7665
S. bulbocastanum	7636		7616		7777
	7637		7738		7778
	7638	*S. medians*	7178		7786
	7641		7619		7795
	7644	*S. megistacrolobum*	3759	*S. sparsipilum*	3533
	7649	*S. michoacanum*	3847	*S. spegazzinii*	3744
	7650		7783		3745
	7651		3740		7195
S. canasense	3059		4048	*S. tarijense*	7523
	3664		4054		7207
	7038		7160		7210
	7142		7163	*S. tuberosum Group Phureja*	4188
	7615		7174		4485
	7716		7176	*S. venturii*	3715
	7720		7707		7627
S. capsicibaccatum	7760		7710	*S. vernei*	7630
	3554		7711		7631
S. chacoense	7211		7712		7789
	3057		7714		7797
	3507		7730	*S. verrucosum*	3939
	3886	*S. mochiquense*	6021		54
	3903	*S. neorossii*	7790		5689
	5849		7628		7091
	5915	*S. okadae*	3761		7213
	5916		7129		7217
	7234		7327		7796
			7620	*S. violaceimarmoratum*	7128
			7625		7611
			7629	*S. x doddsii*	7040
			7775		7148

Resistance was observed within 29 of those accessions, belonging to the species *S. bulbocastanum*, *S. capsicibaccatum*, *S. microdontum, S. mochiquense, S. okadae*, *S.*

pinnatisectum, *S. polyadenium, S. tarijense* and *S. verrucosum* (Table IV.2). There was a strong correlation in the resistance phenotypes observed with both isolates and in the seedling vs. whole plant assays.

Table IV.2: Seedling and whole plant late blight resistance screening results for 29 diploid accessions from the CPC. Late blight resistance was assessed on 25 4-5 week old seedlings (two replicates per test) or 9-10 weeks old selected plants from the accession (two replicates per plant) with the isolates 2006-3928A or 2009-7654A (both 13-A2), respectively. Results were recorded at 8 dpi, using a sliding scale of resistance ranging from 1 = very susceptible to 5 = very resistant for seedling tests and 1 = very susceptible to 9 = very resistant; symptomless plants, for whole plants according to the Malcolmson scale (Cruickshank et al., 1982). The resistance in accession 3762 (denoted with a *) is known to be based on the presence of *Rpi-vnt1.1* only.

Species	CPC accession	Seedling tests with 2006_3928A [1=S to 5=R] Mean of 2 replicates	Whole plant test with 2009_7654A [1=S to 9=R] Mean of 2 replicates
S. bulbocastanum	7636	4	9
	7637	5	-
	7639		9
	7641	5	9
	7642	-	9
	7643	-	9
	7644	4	9
	7645	-	9
	7646	-	9
	7647	-	9
	7650	5	9
	7651	4	9
S. capsicibaccatum	7760	4.5	8.5
S. microdontum	3724	-	9
	3764	-	8.5
S. mochiquense	6021	5	-
S. okadae	7129	5	9
	7625	5	9
	7629	5	9
	3762*	5	
S. pinnatisectum	7521	5	-
	7659	5	-
S. polyadenium	7665	-	9
	7777	4.9	9
	7778	4.4	9
	7786	4.6	8
	7795	3.7	7.5
S. tarijense	7515	5	-
S. verrucosum	54	4	8

To determine if the resistances in these species are based on novel or already characterised resistance genes, a number of complementary assays were performed. In this study I focused only on accessions of *S. okadae* and tested for the presence of *Rpi-vnt1.1* amongst other characterised R genes. The resistance gene *Rpi-vnt1.1* was initially cloned from *S. venturii* and *S. okadae* as well as *S. phureja* accessions and is a homolog of the tomato mosaic virus gene *TM-2(2)* (Foster et al., 2009).

B. Avr-vnt1 is recognised by some *S. okadae* accessions

A set of over 90 *P. infestans* RXLR effectors has been cloned into binary expressions systems to allow the heterologous expression via *Agrobacterium tumefaciens*. With the help of Dr Xinwei Chen, a subset of 82 effectors that includes known *Avr* genes (Table IV.3) such as *Avr-vnt1* (Pel, 2010) was screened on accessions of *S. okadae* including susceptible plants *S. okadae* 7775 and 3761.

Table IV.3: *P. infestans* effectors and effector candidates (PITGs) cloned into binary vector pGRAB and transformed into *A. tumefaciens* strain *Agl1.* Known *Avr* genes are denoted.

ID of PITG	Avr gene	ID of PITG	Avr gene
PITG_04089		PITG_13628-2	
PITG_04314		PITG_13959-1	
PITG_06087		PITG_14736-1	
PITG_06308		PITG_14833-2	
PITG_06478		PITG_(15125)15123-5	
PITG_14371	*Avr3a*	PITG_(16240)16427-1	
PITG_15123		PITG_16737-1	
PITG_15127		PITG_19800-3	
PITG_16294	*Avr-vnt1*	PITG_582	
PITG_18215	*Avr3b*	PITG_2860	
PITG_18670		PITG_4090	
PITG_20300		PITG_4266	
PITG_20303	*Avr-blb2*	PITG_07550-1	
PITG_16663		PITG_07550-8	
PITG_05096		PITG_07550-9	
PITG_08278		PITG_09732-1	
PITG_11484	*Avr10*	PITG_09732-2	
PITG_11507		PITG_09732-3	
PITG_16195		PITG_10232	
PITG_16726		PITG_10540	
PITG_18221		PITG_10654	
PITG_19617		PITG_12731	
PITG_19942		PITG_12737	
PITG_21778		PITG_13093	
PITG_22724		PITG_14443	
PITG_22798		PITG_15110	
PITG_23239		PITG_15278	
PITG_21388.2	*ipiO1*	PITG_16705	
PITG_00366-1		PITG_17063	
PITG_00821-2		PITG_17309.2	
PITG_03192-16		PITG_21740	
PITG_04097-1		PITG_22604	
PITG_04339-1		PITG_22804	
PITG_04388-2		PITG_22922	
PITG_05750-1		PITG_23015	
PITG_07689-5		PITG_23226	
PITG_09585-1		PITG_04085-1	
PITG_9680		PITG_05846	
PITG_10673-7		PITG_20301	
PITG_11383-1		PITG_04145-2	
PITG_13625-7		vir2, 01-29	

The Table IV.4 summarises the effectors triggering HR upon recognition in the infiltrated resistant *S. okadae* accessions 7129, 7625 and 7629.

Table IV.4: *P. infestans* effectors (PITGs) triggering recognition responses in resistant *S. okadae* accessions 7129, 7625 and 7629 after infiltration. Reported are the number of responses / inoculation sites. Known *Avr* genes are denoted.

ID of PITG	Avr gene	Recognition in resistant S. okadae
PITG_16294	*Avr-vnt1*	7129 (9/11) 7625 (7/13) 7629 (9/10)
PITG_20300		7625 (2/4)
PITG_16663		7625 (2/3)
PITG_08278		7625 (2/3)
PITG_11507		7625 (2/3)
PITG_16195		7625 (2/4)
PITG_09732-1		7625 (2/2)
PITG_09732-3		7129 (3/4) 7625 (5/6) 7629 (3/3)
PITG_10540		7629 (2/3)
PITG_10654		7129 (3/4)
PITG_16705		7129 (2/2)
PITG_22922		7629 (2/3)
PITG_04145-2		7625 (2/3) 7629 (2/3)

In at least seven independent replicates with more than 14 individual infiltration sites in total, Avr-vnt1 was recognised reproducibly in *S. okadae* accessions 7129, 7625 and 7629 but not in susceptible plants 7775 or 3761 (Figure IV.1). Other effectors were also recognised in these three resistant accessions and include: for 7129: PITG_09732-3 (3/4); PITG_10654 (3/4); PITG_16705 (2/2); for 7625: PITG_20300 [Avr-blb2-like](2/4); PITG_16663 (2/3); PITG_08278 (2/3), PITG_11507 (2/3); PITG_16195 (2/4); PITG_09732-1 (2/2); PITG_09732-3 (5/6); PITG_04145-2 (3/6); and for 7629: PITG_09732-3 (3/3); PITG_10540 (2/3); PITG_22922 (2/3); PITG_04145-2 (2/3). The data suggest that, in addition to Avr-vnt1, PITG_09732-3 was

also consistently recognised in these three resistant accessions but not in any susceptible plants (Table IV.4).

The *S. okadae* accession 3762 containing *Rpi-vnt1.1* (Hein et al., unpublished) was subjected to effector screenings but did not yield any reproducible responses, and results are not shown in Table IV.4. For example, Avr-vnt1 yielded one phenotypic response out of 11 inoculations. The most consistent responses were observed with PITG_20303 (Avr-blb2 family), where 2 out of 3 sites yielded an HR (data not shown as not reproducible). Unfortunately, the clone 3762 no longer exists due to propagation problems, and I was unable to establish if these results were due to this *S. okadae* clone being not suitable for transient *Agrobacterium tumefaciens* based effector delivery.

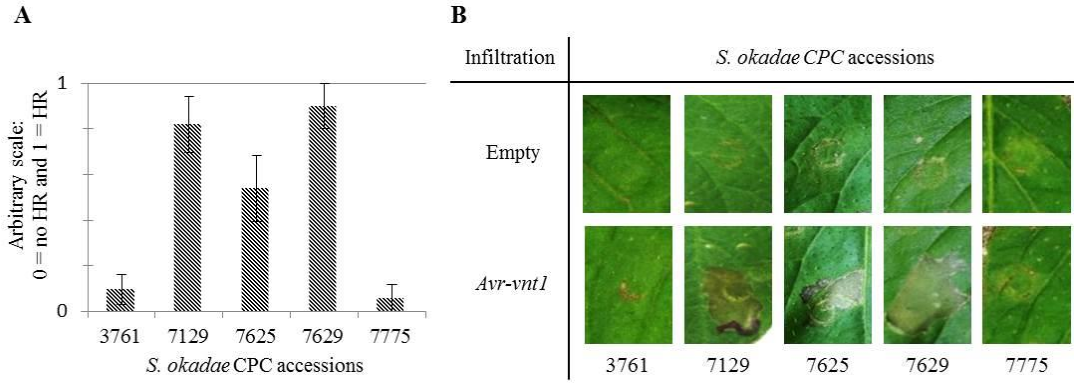

Figure IV.1: Recognition responses following transient, *Agrobacterium tumefaciens*-based expression of *Avr-vnt1* in *S. okadae*. Accessions resistant to *P. infestans* genotype 13-A2, 7129, 7625 and 7629, yield a visible response whereas the susceptible accessions 3761 and 7775 yield no specific response if compared to empty vector control. (A) Graph representing phenotypic response at the Avr-vnt1 infiltration sites from at least three independent replicates. Plants were scored at 5 dpi. A score of zero represents no HR and a score of one indicates that at least half the infiltrated leaf area responded with a cell death response. (B) Pictures of the infiltration sites of the empty vector control and Avr-vnt1 visualised under white light at 5 dpi. Transient

expressions were performed by infiltration of *A. tumefaciens* strain Agl1, at an OD_{600} of 0.2.

2. Allele mining and diagnostic RenSeq confirm that *S. okadae* accessions contain *Rpi-vnt1.1*

Rpi-vnt1.1 gene specific PCR primers were designed and utilised to ascertain if the *S. okadae* accessions 7129, 7625 and 7629 contain the 2676bp long gene *Rpi-vnt1.1* (Foster et al., 2009) that is also present in *S. okadae* accession 3762 (Hein et al., unpublished). PCR was conducted on genomic DNA of six *S. okadae* accessions including the susceptible accessions 7775 and 7620. Bands of the expected ~2.6Kb size were observed by gel electrophoreses for all the samples (Figure IV.2).

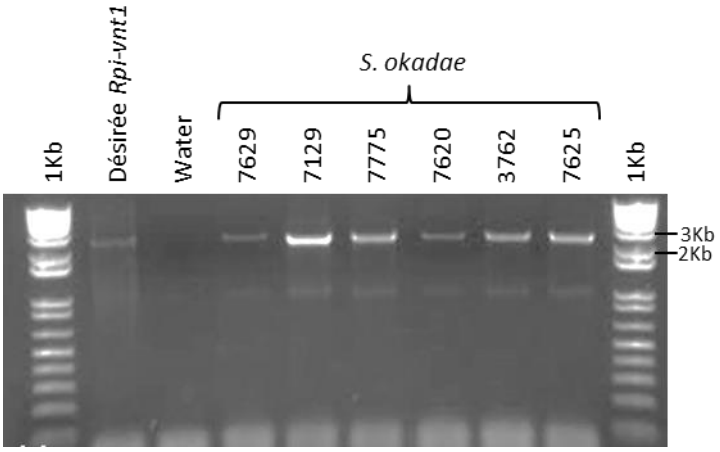

Figure IV.2: Full length *Rpi-vnt1* PCR screen of the *S. okadae* accessions. DNA from a *Rpi-vnt1* transgenic potato plant (*cv.* Désirée) was used a positive control and water as negative control. Picture was taken under UV light after electrophoresis on a 1% agarose gel supplemented with 0.5µg/mL ethidium bromide.

S. okadae accessions 7620 and 7775 are susceptible and were not investigated further in this study. *S. okadae* 3762 was no longer available in the glasshouse as the material coud not be efficiently propagated, and further studies on this accessions were not possible. PCR products from resistant accessions 7129, 7625 and 7629 were cloned and Sanger sequenced

to establish the sequences of individual clones. Alignment of PCR product sequences with *Rpi-vnt1.1* indicates that all three accessions contain a sequence identical to *Rpi-vnt1.1* alongside additional gene variations and truncated sequences (Figure IV.3).

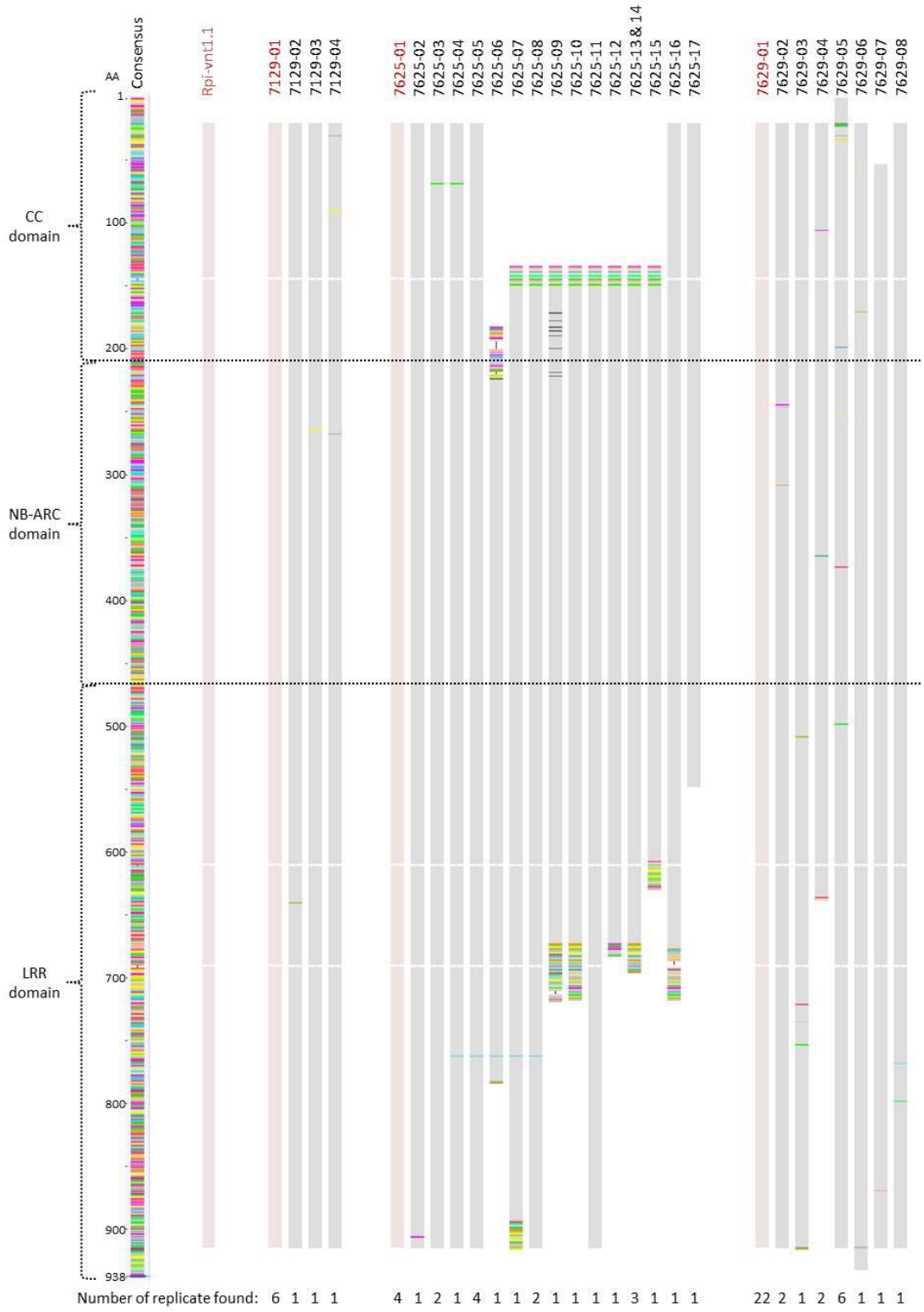

Figure IV.3: *Rpi-vnt1.1* allele mining in *S. okadae* accessions. In total 9, 26 and 36 *Rpi-vnt1-like* genes have been amplified and sequenced from the *S. okadae* accessions 7129, 7625 and 7629, respectively. Nucleotide sequences were translated and the amino acid sequences aligned using *Rpi-vnt1.1* as a reference. Sequences identical to

Rpi-vnt1.1 are shown in red. The sequence redundancy for each clone is shown below the alignment.

RenSeq-based sequence analysis was conducted to corroborate the allele mining results and to establish weather RenSeq could be used as a diagnostic tool for validating the presence of functional NB-LRR genes. With the help of Dr Miles Armstrong, genomic potato DNA samples from *S. okadae* accessions 7129 and 7625 were indexed with Illumina sequencing compatible adapters, enriched for NB-LRR genes using the new probe library described in chapter III, and sequenced on a single lane of Illumina MiSeq. Each sample took a twelfth of the MiSeq lane. Following quality control, 1,814,975 paired-end reads were obtained for *S. okadae* accession 7129 and 1,518,349 for 7625. Mapping against the sequenced potato clone DM, which has 704 NB-LRRs with known positions on chromosomes 1 to 12 (Jupe et al., 2013) was conducted at 0.5%, 1%, 5% and 10% mismatch rates by Dr Katie Baker. At 0.5% and 1% mismatch rates the systematic differences between *S. okadae* and the sequenced S*. phureja* (DM) were apparent and a maximum of 6.49% of all reads could be mapped of which more than 50% were on target. However, when allowing for a 5% or 10% mismatch rate, more than 46% or 70% of all reads could be mapped, respectively. Furthermore, the on-target rate increased to a maximum of 69.5% and mean coverage of NB-LRRs reached 108x (Table IV.5). Importantly, more of the 704 NB-LRR reference genes from DM were covered by reads from *S. okadae* accessions with conditions allowing for 5% or higher mismatch rates (Figure IV.4A, Figure IV.5A and Table IV.6) indicating that the enrichment was successful.

Sequences derived from 7129 and 7625 were also mapped to a reference set of 12 characterised potato late blight NB-LRR sequences including *R1, R2, R2-like, Rpi-abpt, Rpi-blb3, R3a, R3b, Rpi-blb1, Rpi-pta1, Rpi-sto1, Rpi-blb2* and *Rpi-vnt1.1* in a dRenSeq analysis. At 1% mismatch rate, only functional *Rpi-vnt1.1* was completely represented by RenSeq reads (Figure IV.4B and Figure IV.5B). Similar specific results were observed at 0.5% mismatch

rate but not at 5% or 10% (Figure IV.6). Indeed, at 5% and 10% mismatch rates, the mean read coverage of *Rpi-vnt1.1* was comparable to other characterised R genes (Figure IV.6).

Table IV.5: RenSeq reads mapped to DM genome v4.03 or a reference set of 12 R genes at various mismatch rates (%MM). The resulting alignments were intersected (+/-1000bp) against the 704 R genes from DM with known locations on chromosomes 1-12 to give the proportion of on target reads. The on target reads were then assessed for mean read coverage against the 704 genes, whilst for the 12 R gene set all the mapped reads were used to calculate the read depth.

CPC	% MM	Reads mapped to DM genome v4.03					Reads mapped to 12 functional NB-LRRs		
		Total	% Mapped	On target	% On target	Mean coverage (x)	Total	% Mapped	Mean coverage (x)
7129	0.5	87842	2.42	33585	38.23	1.93	1386	0.04	9.07
	1	203384	5.60	108583	53.39	6.49	2034	0.06	13.36
	5	1685852	46.44	1147209	68.05	72.83	50442	1.39	328.75
	10	2554646	70.38	1696516	66.41	108.23	234404	6.46	1568.62
7625	0.5	85054	2.80	39880	46.89	2.22	736	0.02	4.57
	1	197172	6.49	118332	60.01	6.83	1214	0.04	7.26
	5	1460566	48.10	1015151	69.5	62.63	60442	1.99	384.19
	10	2170588	71.48	1472915	67.86	91.58	256646	8.45	1683.09

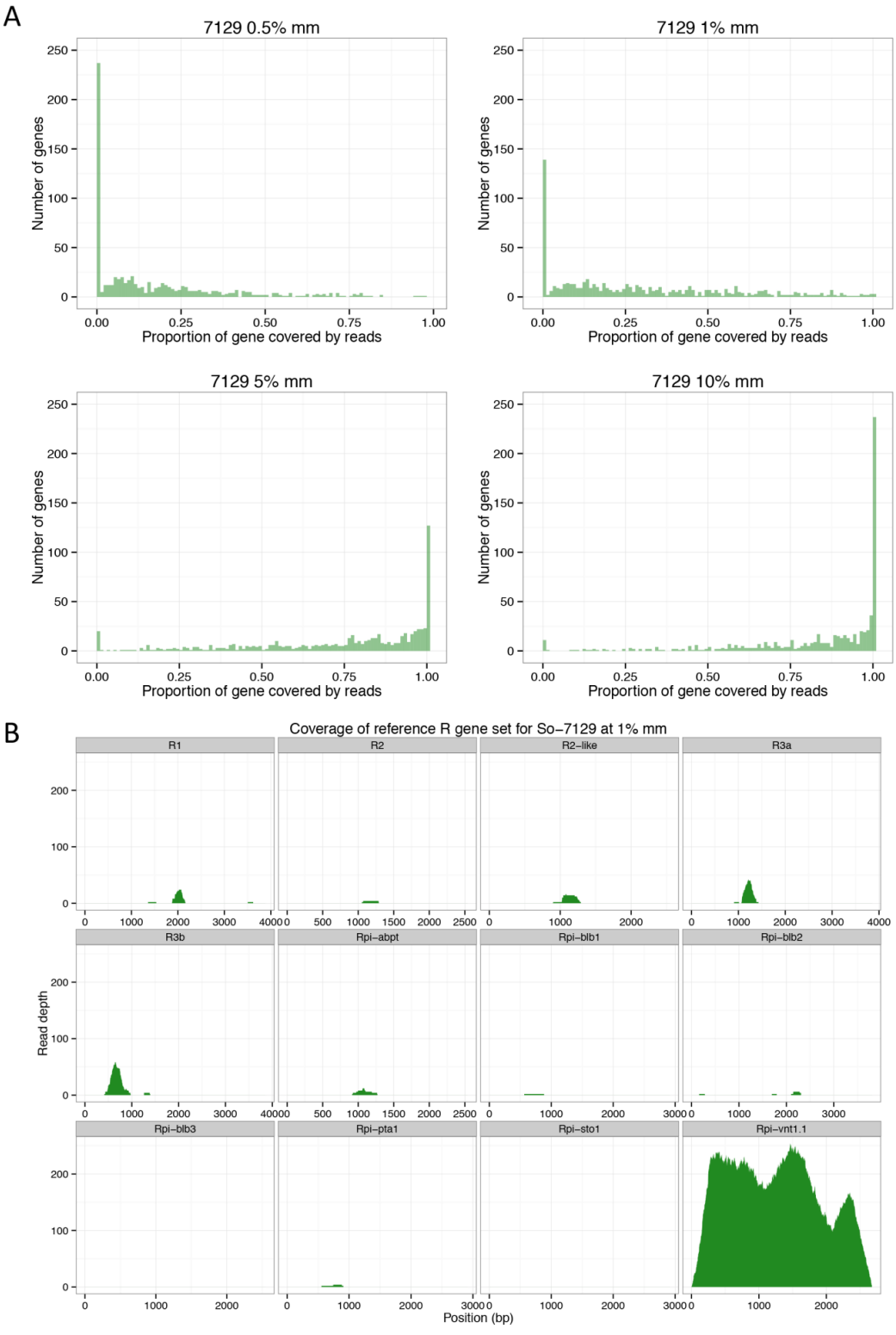

Figure IV.4: RenSeq analysis for *S. okadae* accession 7129. A) The number of 704 R genes from DM with known locations on chromosomes 1-12 that are not covered (0.00), partially covered or fully covered (1.00) following RenSeq analysis in *S. okadae*

accession 7129 is shown. Mismatch rates (%mm) ranging from stringent 0.5% or 1% to more relaxed 5% or 10% are displayed. B) The read depth and coverage of 12 functional R genes with homologous sequences isolated from *S. okadae* accession 7129 following RenSeq analysis and mapping under stringent conditions (1% mismatch rate) are depicted.

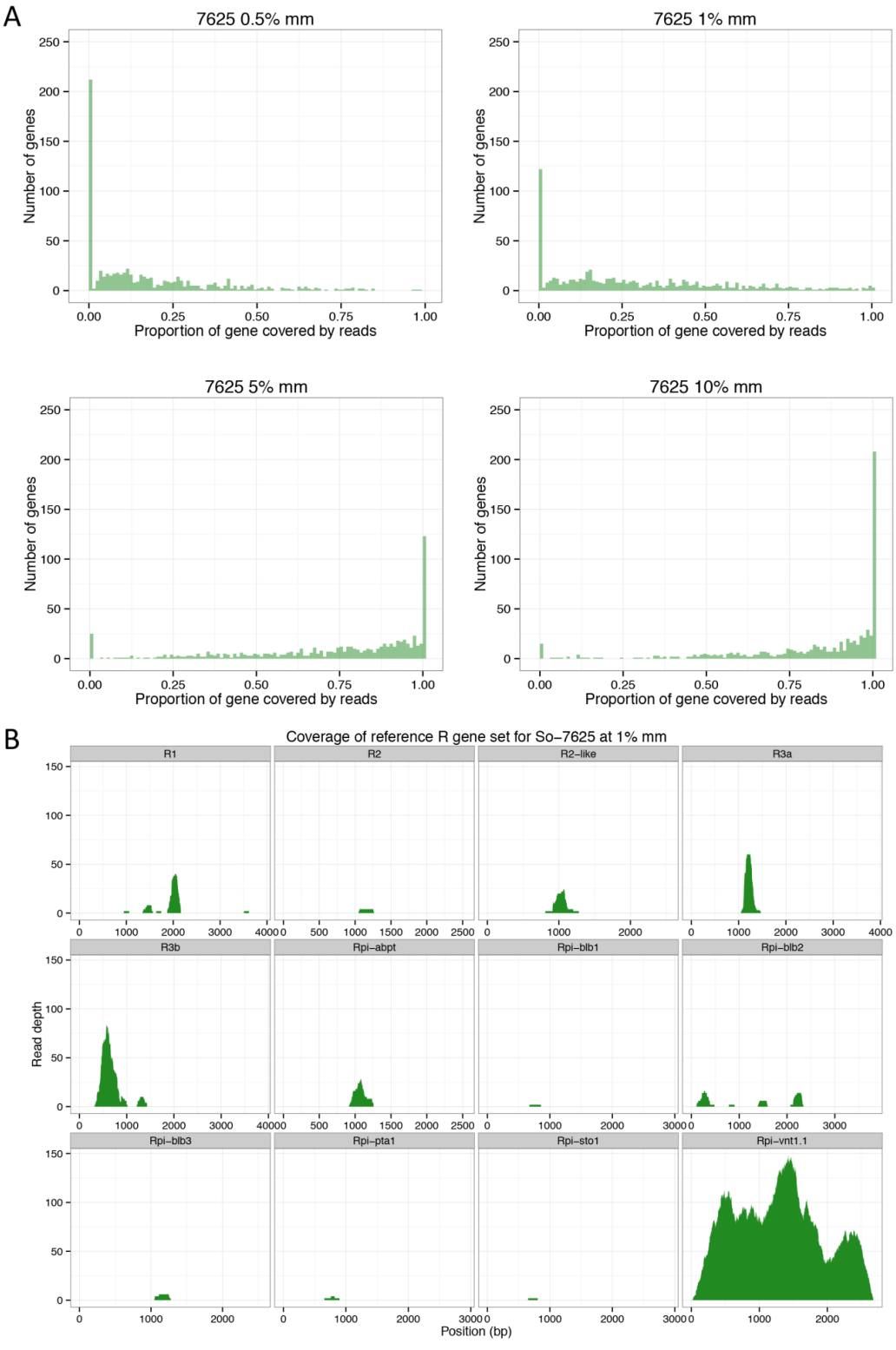

Figure IV.5: RenSeq analysis for *S. okadae* accession 7625. A) The number of 704 R genes from DM with known locations on chromosomes 1-12 that are not covered (0.00), partially covered or fully covered (1.00) following RenSeq analysis in *S. okadae*

accession 7625 is shown. Mismatch rates (%mm) ranging from stringent 0.5% or 1% to more relaxed 5% or 10% are displayed. B) The read depth and coverage of 12 functional R genes isolated from *S. okadae* accession 7625 following RenSeq analysis and mapping under stringent conditions (1% mismatch rate) are depicted.

Table IV.6: RenSeq reads mapped to DM genome v4.03 at 0.5, 1, 5 and 10% mismatch rates (%MM). The resulting alignments were cross-referenced against the 704 R genes from DM with known locations on chromosomes 1-12 to determine how many R genes were covered extensively (≥95%), completely (100%), minimally (≤5%) or not at all (0%). For example, in *S. okadae* 7629 and with a 5% mismatch rate, 20 NB-LRRs were not present at all, 22 NB-LRRs where minimally covered, 231 NB-LRRs were extensively covered and 127 NB-LRRs where completely covered, out of the 704 DM NB-LRRs used as reference.

Sample	% MM	Number of genes with % coverage			
		0%	≤5%	≥95%	100%
7129	0.5	236	278	3	0
	1	138	167	14	3
	5	20	22	231	127
	10	11	12	340	237
7625	0.5	211	259	3	0
	1	121	156	15	3
	5	25	26	200	123
	10	15	17	318	208

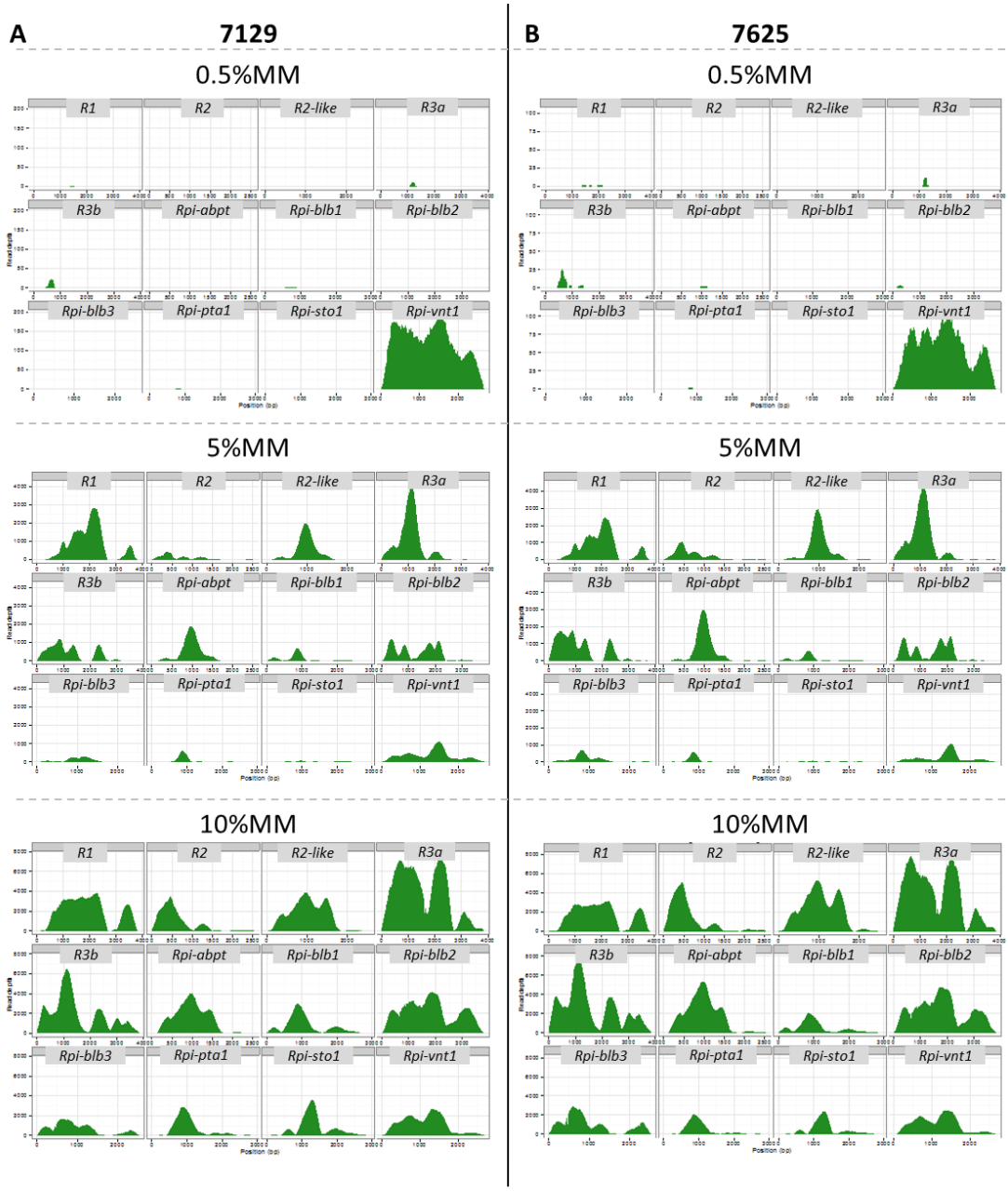

Figure IV.6: dRenSeq analysis for *S. okadae* accession 7129 and 7625 under different mapping stringencies. The read depth and coverage of 12 functional R genes with reads obtained from (A) *S. okadae* accession 7129 and (B) 7625 following RenSeq analysis is depicted. Read mapping was conducted under very stringent conditions (0.5% mismatch rate [%MM]) and relaxed conditions (5% and 10% mismatch rate).

Importantly, dRenSeq was also applied to resistant *S. okadae* accession 3762 (containing *Rpi-vnt1.1*) and susceptible *S. okadae* 3761 (without functional *Rpi-vnt1.1*) to validate the

concept and to discern between resistant and susceptible plants from the same species. Included were also a pool of 20 resistant and 20 susceptible plants that are derived from a cross between both accessions (Figure IV.7). Dr Katie Baker conducted the mapping against the sequenced potato clone DM at 0.5%, 1%, 5% and 10% mismatch rates (Table IV.7). As observed for Table IV.5, the levels of gene coverage increased with allowing for higher mismatch rates. At 0.5% and 1% mismatch rates the systematic differences between *S. okadae* and S*. phureja* (DM) were apparent and a maximum of 9.01% of all reads could be mapped of which more than 50% were on target. However, when allowing for a 5% or 10% mismatch rate, more than 49% or 75% of all reads could be mapped, respectively. Furthermore, the on-target rate increased to a maximum of 68.27% and mean coverage of NB-LRRs reached 229.98x (Table IV.7). At a mismatch rate of either 0.5% or 1%, full-length *Rpi-vnt1.1* was recovered from accession 3762 and the resistant pool (Figure IV.7). However, an *Rpi-vnt1.1*-like sequence with a truncated 5' end, compared to the functional gene, was recovered from both the susceptible accession 3761 and the susceptible pool. Indeed, the lack of sequence conservation in this region was consistently detected in both susceptible samples (Figure IV.7).

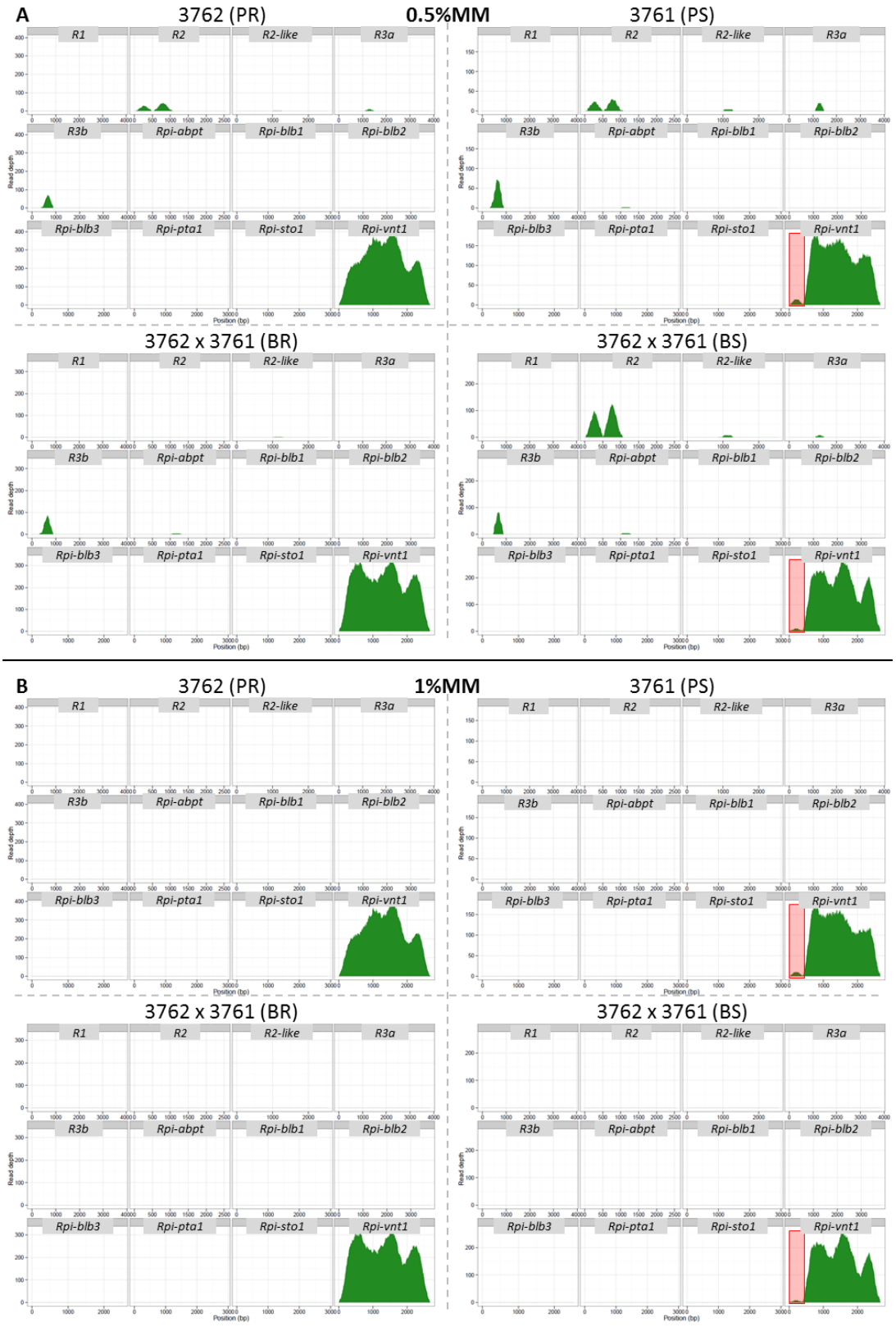

Figure IV.7: dRenSeq analysis for resistant and susceptible *S. okadae* accession and bulked progeny. The read depth and coverage of 12 functional *R* genes with homologous sequences following RenSeq analysis and mapping under stringent

conditions are depicted in A) 0.5% mismatch rate (0.5% MM) and B) 1% mismatch rate (1%MM). Sequences were isolated from *S. okadae* accessions including 3762 carrying *Rpi_vnt1.1* (Parent Resistant called PR), 3761 (Parent Susceptible called PS), bulk of 20 resistant plants (BR) derived from a cross between 3762 and 3761 and bulk of 20 susceptible plants (BS) derived from a cross between 3762 and 3761. Outlined in red is the part of *Rpi-vnt1* not present in the susceptible material (very low read deapth).

Table IV.7: RenSeq reads mapped to DM genome v4.03 or a reference set of 12 R genes at various mismatch rates (%MM). The resulting DM alignments were intersected (+/-1000bp) against 704 R genes from DM with known locations on chromosomes 1-12 to give the proportion of on target reads. The on target reads were then assessed for

mean read coverage against the 704 genes, whilst for the 12 R gene set all the mapped reads were used to calculate the read depth.

CPC	% MM	Reads mapped to DM genome v4.03					Reads mapped to 12 functional NB-LRRs		
		Total	% Mapped	On target	% On target	Mean coverage (x)	Total	% Mapped	Mean coverage (x)
3762	0.5	174308	3.18	56341	32.32	3.62	2514	0.05	17.02
	1	444416	8.10	249047	56.04	16.02	2748	0.05	18.60
	5	2803274	51.11	1913685	68.27	123.05	72136	1.32	487.94
	10	4219454	76.93	2838323	67.27	182.40	384018	7.00	2596.19
Res. pool	0.5	179834	2.44	67424	37.49	4.33	2302	0.03	15.57
	1	529446	7.19	309808	58.52	19.93	2464	0.03	16.66
	5	3685852	50.03	2419464	65.64	155.49	91688	1.24	619.93
	10	5593852	75.92	3581321	64.02	229.98	468144	6.35	3163.43
3761	0.5	249026	3.92	67861	27.25	4.36	1080	0.02	7.31
	1	572882	9.01	288364	50.34	18.55	1290	0.02	8.73
	5	3280916	51.60	2131810	64.98	137.09	84938	1.34	574.61
	10	4867230	76.54	3127877	64.26	201.02	437200	6.88	2956.29
Sus. pool	0.5	170244	2.62	57659	33.87	3.70	1470	0.02	9.95
	1	476846	7.35	268122	56.23	17.24	1888	0.03	12.78
	5	3189788	49.14	2065139	64.74	132.72	74080	1.14	500.86
	10	4845936	74.66	3062376	63.19	196.67	395862	6.10	2675.09

3. *S. okadae* accessions contain additional resistance that is independent of *Rpi-vnt1.1*

Selected *S. okadae* accessions were screened with five additional *P. infestans* isolates that display broad race specificity (Table II.2). Importantly, the isolate EC1, which overcomes *Rpi-vnt1.1* resistance, was included to discern between resistances that are exclusively based on the presence of *Rpi-vnt1.1*. The clone Rpi-vnt1.1_R6, which is derived from the cross

between *S. okadae* accessions 3762 (containing *Rpi-vnt1.1*) and 3761 (susceptible), was used as a control.

In line with previous results, the clone Rpi-vnt1.1_R6 was resistant to the blue 13 isolate 2009-7654A and other isolates but susceptible to EC1 (Table IV.8). The *S. okadae* accession 7775 was susceptible to the blue 13 isolate but partially resistant to EC1. The three *S. okadae* accessions that recognise Avr-vnt1 (Figure IV.1), however, were resistant to all isolates including EC1 (Table IV.8 and Figure IV.8).

Table IV.8: Late blight screen of five diploid *S. okadae* accessions from the CPC. The isolate names and genotypes are shown where known. The blight tests were performed on detached leaves using different isolates of *P. infestans*. Results were scored at 8 dpi, from 1 = susceptible to 5 = resistant; symptomless leaf. The scores shown are the average of at least two independent replicates.

CPC accession number	Species or cultivars	*P. infestans* isolates (genotype)					
		2009-7654A (13 A2)	2010-7822 (6A1)	2010-7814 (23A1)	2010-8122D (8 2 A1)	2010-7838A (Misc')	EC1 (non-characterised)
3761	*S. okadae*	1.0	1.5	4.0	2.0	1.5	-
Rpi-vnt1.1_R6	*JHI cross*	5.0	5.0	5.0	5.0	5.0	1.0
7129	*S. okadae*	5.0	5.0	5.0	5.0	5.0	5.0
7625	*S. okadae*	5.0	5.0	5.0	5.0	5.0	4.0
7629	*S. okadae*	5.0	5.0	5.0	5.0	5.0	5.0
7775	*S. okadae*	1.0	-	-	-	-	3.0

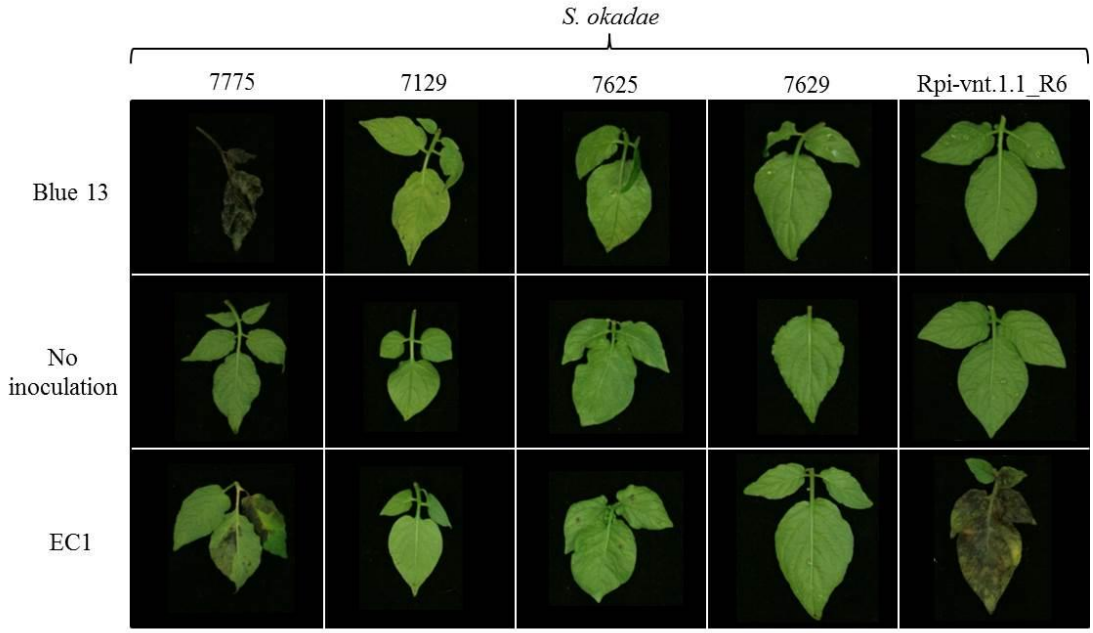

Figure IV.8: Late blight screen of *S. okadae* accessions with EC1, a *Rpi-vnt1.1* race specific isolate of *P. infestans,* and blue 13. Isolates of *P. infestans* were drop-inoculated on detached leaves and symptoms assessed at 8 dpi. The *S. okadae* clone 3762-R6 has been independently characterised and only contains *Rpi-vnt1.1,* and was used as a control.

This provides evidence that these accessions, unlike clone Rpi-vnt1.1_R6, carry at least one additional, novel resistance gene that functions independently of *Rpi-vnt1.1*.

4. dRenSeq on the potato population B3C1HP shows that late blight resistance is most likely based on the recently cloned resistance gene R8.

Following the success of the dRenSeq on the *S. okadae* samples mentioned above, a similar work flow was applied to the potato population B3C1HP (Li et al., 2015), to assess the presence of known functional NB-LRR genes in the resistant material (Chapter III). With the help of Dr Miles Armstrong, four genomic potato DNA samples (parent resistant, parent susceptible, bulk resistant containing 27 resistant progeny and bulk susceptible containing 27 susceptible progeny) were indexed, enriched for NB-LRR genes, and sequenced. Following quality control, 1,286,701 paired-end reads were obtained for the parent resistant,

1,582,117 for the parent susceptible, 1,866,832 for the bulk resistant and 1,899,506 for the bulk susceptible. Sequences derived from the four DNA samples were mapped to a reference set of 14 characterised potato late blight NB-LRR sequences in a dRenSeq analysis, including *R1, R2, R2-like, Rpi-abpt, Rpi-blb3, R3a, R3b, R8, R9a, Rpi-blb1, Rpi-pta1, Rpi-sto1, Rpi-blb2* and *Rpi-vnt1.1*. The nucleotide sequences for R8 and R9a (both unpublished), were kindly provided by Dr Jack Vossen from Wageningen University. Following the stringent mapping of the reads at 0.5% mismatch rates, only the functional *R8* was completely represented by RenSeq reads in the resistant parent and resistant bulk (Figure IV.9A and 9C). The susceptible material did not show full coverage of any of the known *R* genes (Figure IV.9B and 9D).

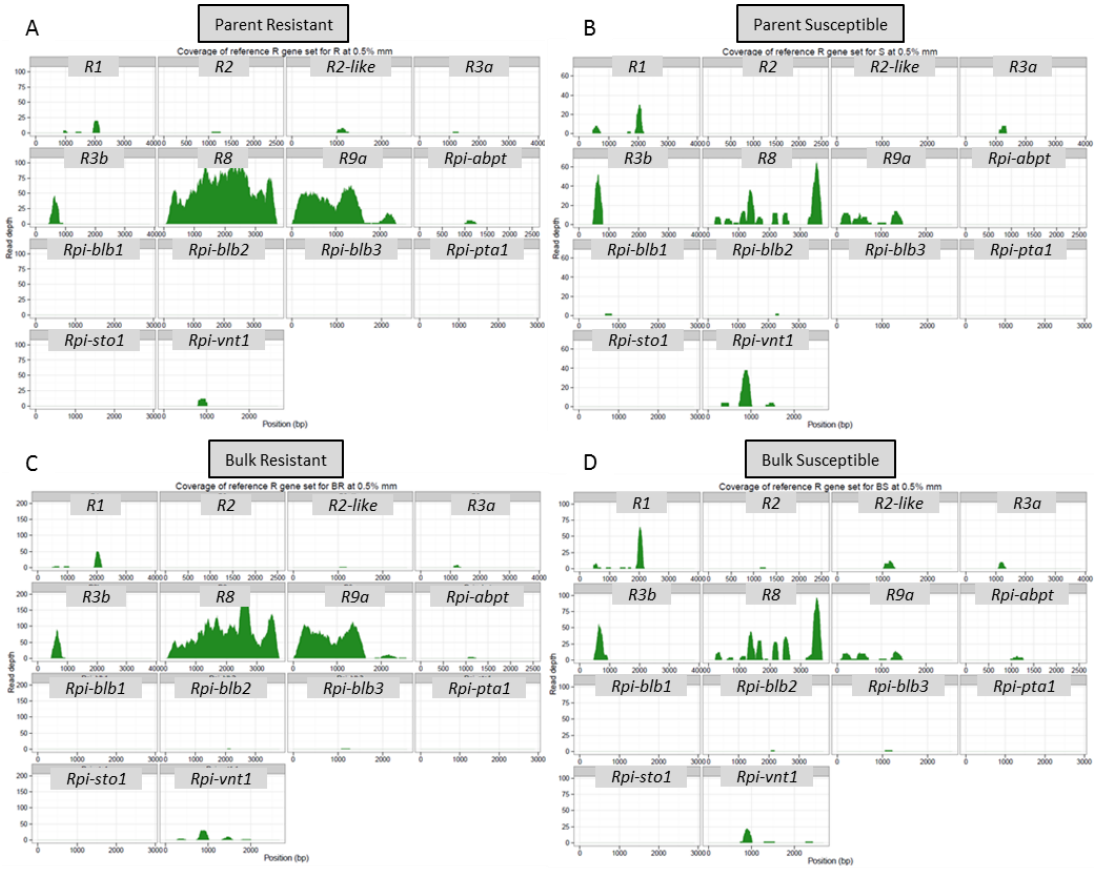

Figure IV.9: dRenSeq analysis for resistant and susceptible parents and bulked progeny of the diploid potato population B3C1HP (Li et al., 2015). The read depth and coverage of 14 functional R genes with homologous sequences isolated from the diploid potato population B3C1HP (A) resistant parent, (B) susceptible parent, (C) bulk of 27 resistant plants from B3C1HP and (D) bulk of 27 susceptible plants from B3C1HP following RenSeq analysis and mapping under stringent conditions (0.5 % mismatch rate) are depicted.

III. Discussion

Potato production is constantly threatened by late blight disease and the need for fast and reliable diagnostic *R* gene tools is apparent. Effector-omics has proven useful for breeding and the identification of orthologous R genes in wild species (Vleeshouwers et al., 2008; Vleeshouwers and Oliver, 2014; Lenman et al., 2016). However, for this system to be successful, a detailed knowledge of the recognised effector is required alongside responsive

plants that yield a reproducible recognition response upon transient effector expression. We have obtained reproducible Avr-vnt1 recognition responses in *S. okadae* accessions 7129, 7625 and 7629 (Figure IV.1) but not for 3762 that contains the cognate R gene *Rpi-vnt1.1*. The latter proved non-responsive to the transient *Agrobacterium*-based expression system. In addition, PITG_09732-3 was recognised in these three accessions but not in any susceptible accession and could provide a clue for the basis of the *Rpi-vnt1.1* independent resistance observed in these plants. However, without following this up in a segregating population, it would be premature to further speculate. In particular, the promiscuous recognition events in *S. okadae* 7625 need further investigation.

In line with the Avr-vnt1 recognition, PCR-based allele mining and Sanger sequencing confirmed the presence of *Rpi-vnt1.1* in *S. okadae* accessions 7129, 7625 and 7629 (Figure IV.3). A similar approach has been utilised successfully to identify orthologous genes in wild potato species (Lokossou et al., 2009; 2010). A PCR-based screening for full-length *R* genes alone could, however, be prone to false-positives as shown with the susceptible *S. accessions* 7775 and 7620 (Figure IV.2). However, this conclusion is based on the assumption that 7775 and 7620, being susceptible *S. okadae* accessions, do not carry a full length *Rpi-vnt1* gene, as no cloning have been performed in these accessions. Indeed, the cloning and sequencing of PCR products, which is required to discriminate highly similar sequences (Figure IV.3), is time consuming and renders this process low to medium throughput.

This study has shown that mapping RenSeq reads with stringent mismatch rates against reference R genes, results in a quick and easy way to screen plants for the presence or absence of known R genes (Figures 5, 6, 7, 8 and 9).

The experimental design allowed to combine 12 individual plants for the RenSeq analysis and simultaneously gain knowledge for about 755 NB-LRR genes per plant (12 x 755 = 9060 genes in total). A detailed analysis was conducted initially for 12 previously identified NB-LRRs per

plant (12 x 12 = 144 genes in total). However, the sequence information for additional, currently unpublished functional NB-LRR such as R8 and R9a could be incorporated easily for a dRenSeq (Figure IV.9) and highlights the scalability of the approach. To achieve the same in-depth analysis through Sanger sequencing, 71 clones were sequenced for a single gene (*Rpi-vnt1.1*) in three accessions of *S. okadae* (Figure IV.3). We therefore assume that a sequence analysis of about 20 clones is required per gene and individual plant. To investigate the presence/absence of 12 functional genes in 12 individual plants in more detail by Sanger sequencing, more than 2880 clones would be required. The costs would be prohibitive and the process extremely labour intensive. Indeed, Sanger sequencing was used as a control to validate dRenSeq and Effectoromics. The work demonstrates that dRenSeq has now superseded Sanger sequencing-based allele mining approaches and provides a cheap, reliable and fast alternative.

Indeed, DRenSeq is specific enough that it could distinguish between functional *Rpi-vnt1.1* in resistant accessions and its homologs in susceptible accessions as well as bulks (Figure IV.7). As such, dRenSeq could also be used for allele mining under various stringent mapping conditions and also aid evolutionary studies. Importantly, the obtained RenSeq sequence from plants that do contain novel resistances can subsequently be used as a reference in a bulked-segregant analysis if genetic crosses can be achieved (Jupe et al., 2013). Therefore, sequence data can be used to answer different biological questions.

Interestingly, the *S. okadae* accessions 7129, 7625 and 7629 all contain functional *Rpi-vnt1.1* as demonstrated by effector recognition, allele mining and, in the case of 7129 and 7625, DRenSeq. However, they also contain a resistance that operates independent of *Rpi-vnt1.1* as demonstrated by additional late blight screening (Figure IV.8). The clone Rpi-vnt1.1_R6 carries *Rpi-vnt1.1* and is, as expected, resistant to Blue13 but susceptible to the isolate EC1

(Foster et al 2009), whereas 7129, 7625 and 7629 were all resistant to both isolates (Figure IV.8).

The use of DRenSeq on the potato population B3C1HP (Li and al., 2015) suggested that its resistance to late blight is related to R8 (Figure IV.9). This shows the power of the dRenSeq in diagnosing known R genes in uncharacterised material.

Future efforts to identify resistances towards major pathogens in germplasm collection can quickly identify plants that contain novel resistances by taking advantage of target enrichment and sequencing technologies. For example, a combination of late blight screening that includes isolates with a broad virulence spectrum followed by dRenSeq could be utilised to first prioritise plants that could subsequently be subjected to effector-omic analysis prior to a detailed genetic study. In breeding programs, dRenSeq (or similar enrichment strategies for additional genes) could be utilised to aid R gene pyramiding and/or to follow multiple important traits on a sequence-based level.

Chapter V: Identification of the *P. infestans* effector *Avr-Ph3*

This chapter details the identification of *Avr-Ph3* candidates. *Rpi-Ph3* has recently been cloned. However, the cognate *P. infestans* effector that triggers the plant resistance upon perception by *Rpi-Ph3* remains elusive. Thus, the aim of this study was to identify *Avr-Ph3*. To date, all recognised *P. infestans* effectors identified are characterised by a canonical RXLR domain. We therefore rationalised that the cognate effector *for Rpi-Ph3* is also most likely a RXLR-type effector. The screening for candidate effector involved transient expression of a set of 96 cloned RXLR effectors, in different tomato lines containing either no resistance gene, *Rpi-Ph2* or *Rpi-Ph3*. However, transient expression in tomato plants proved to be more difficult than in potato or *N. benthamiana*. The first step of this analysis was thus to establish a suitable transient expression system in tomato. The most reproducible responses were achieved by transiently delivering candidate effectors through Potato Virus X (PVX) with the help of *Agrobacterium tumefaciens* strain GV3101. Once the GV3101-PVX system was established and the effector set re-cloned accordingly, the effector screen in the different lines of tomato identified three families of effectors as potential *Av-Ph3* candidates. Further testing through co-infiltration in *N. benthamiana* of those candidates revealed that only PITG_23015 and PITG_23226 were consistently recognised by Rpi-Ph3. Finally, this study assessed the virulence function of the different *Avr-Ph3* candidates through gain of *P. infestans* pathogenicity upon transient expression.

I. Introduction

The biggest threat to potato and tomato productions world-wide is late blight disease caused by the oomycete pathogen *Phytophthora infestans*. Resistances found in some wild Solanaceae species provide environmentally-benign means of restricting late blight

infections. In general, most plants are resistant to the majority of pathogens. This is due to the recognition of conserved pathogen motifs called pathogens associated molecular patterns (PAMPS), by plant recognition receptors (PRRs) (Jones and Dangl, 2006; Hein et al., 2009; Spoel and Dong, 2012). However, some adapted pathogens have co-evolved and secrete molecules referred to as effectors into plants to perturb this recognition, and thus promote virulence (Hein et al., 2009; Nowicki et al., 2012; Spoel and Dong, 2012). Plant resistance (*R*) gene products function by directly or indirectly recognising these effectors and inducing a resistance response to halt further pathogen ingress. The largest family of plant *R* gene products encodes for nucleotide-binding, leucine-rich repeat proteins known as NB-LRR (Jupe et al., 2012). Recognised effectors are referred to as *avirulence* proteins (AVR). Their recognition often results in a hypersensitive response (HR), a form of programmed cell death (PCD) (Dangl and Jones, 2001). To date, all recognised *P. infestans* effectors identified are characterised by a canonical RXLR domain (Armstrong et al., 2005; Hein et al., 2009; Raffaele et al., 2010; Cooke et al., 2012). This has led to the development of effectoromics (Vleeshouwers et al., 2008 and 2011; Vleeshouwers and Oliver; 2014) to elucidate the molecular components from the pathogen that elicits the immune response upon perception. This knowledge is essential to predict the potential durability of resistances deployed (Birch et al., 2008).

In tomato, several commercial lines carry the *Rpi-Ph3* gene. As mentioned in Chapter III, this gene resides on chromosome 9 and provides resistance towards many US isolates of *P. infestans* (Chunwongse et al., 2002; Zhang et al., 2013; 2014). The tomato genome has been sequenced (TGC, 2012) and recent advances have been made in its genome annotation (Jupe et al., 2013; Andolfo et al., 2014). This gives a better insight on the molecular aspects of tomato resistances. As a result, Rpi-Ph3 was first finely mapped to chromosome 9 (Zhang

et al., 2013), then cloned (Zhang et al., 2014). However, the cognate *P. infestans* effector that triggers the plant resistance upon perception by *Rpi-Ph3* remains elusive.

Objective of this chapter:

The aim of this chapter was to establish a robust, transient expression system of effectors to screen an association panel of tomato lines to identify *Avr-Ph3*. This association panel is formed of susceptible lines, *Rpi-Ph3* containing lines and *Rpi-Ph2* containing lines. Collaborators Dr. Véronique Lefebvre and René Damidaux from INRA Avignon, France, kindly provided all those lines. The second aim was to confirm candidate *Avr-Ph3* genes by co-infiltration with the cloned *Rpi-Ph3* resistance gene (provided by Dr. Jack Vossen, Wageningen University) in *N. benthamiana*. Lastly, the role of *Avr-Ph3* candidates in *P. infestans* pathogenicity was to be assessed upon transient expression of candidates in *N. benthamiana*.

II. Results

1. Effector screen to identify *Avr-Ph2* and *Avr-Ph3*

A. Establishment of a suitable delivery system in tomato

The James Hutton Institute hosts a library of 96 *P. infestans* RXLR effectors cloned into a gateway compatible expression systems. Before screening all effectors in different tomato lines, a preliminary test was performed to identify the most robust delivery system for tomato. Initially, all effectors were available in the binary expression vector pGRAB and transformed in *A. tumefaciens* strain Agl1. However, the localised transient expression of negative controls empty pGRAB (pGRAB:empty), pGRAB expressing red fluorescent protein tdTomato (tdT) and *P. infestans* Inf1 that is typically recognised in *Nicotiana* species but not tomato with this system, yielded non-reproducible results in tomato (Table V.1). Different OD_{600} ranging from 0.1, 0.3 to 0.5 were assessed in two independent replicates with four or

six plants, respectively. In many cases, non-specific chlorosis was observed in the inoculated areas, whereas all the infiltrations performed in parallel in *N. benthamiana* yielded the expected phenotypes (Table V.1).

Table V.1: Infiltration results of the Agl1-pGRAB transient expression test in tomato *cv.* Moneymaker (MM) and *Nicotiana benthamiana* (*N.benth*). Six infiltrations per leaflets were performed (three on each side of the main vein) and the different positions of infiltration were recorded, x being the closest to the petiole and z the closest to the apex of the leaves. Phenotypic responses were scored from 0 = no response to 3 = strong HR at 3dpi. Those scores are also colour coded throughout the table, from white (=0) to red (=3). Different OD_{600} were tested and reported in this table.

OD: 0.1	Independant replicate 1													Independant replicate 2													
Plant	*N. benth*			MM 1			MM 2			MM 3			MM 4			*N. benth*			MM 1			MM 2			MM 3			MM 4			MM 5			MM 6		
Position	x	y	z	x	y	z	x	y	z	x	y	z	x	y	z	x	y	z	x	y	z	x	y	z	x	y	z	x	y	z	x	y	z	x	y	z
Empty	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
tdt1	0	0	0	0	2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1
Inf1	2	2	2	0	0	0	0	0	0	0	0	0	0	0	0	2	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0
OD: 0.3	Independant replicate 1													Independant replicate 2													
Plant	*N. benth*			MM 1			MM 2			MM 3			MM 4			*N. benth*			MM 1			MM 2			MM 3			MM 4			MM 5			MM 6		
Position	x	y	z	x	y	z	x	y	z	x	y	z	x	y	z	x	y	z	x	y	z	x	y	z	x	y	z	x	y	z	x	y	z	x	y	z
Empty	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2	1	2	1	2	1	2	0	1	0	0	0	0	0	0
tdt1	0	0	0	3	3	0	0	0	0	3	3	0	2	0	0	0	0	0	1	0	0	1	2	0	0	3	1	2	3	2	0	0	0	0	0	1
Inf1	2	2	2	0	0	0	0	0	0	0	3	1	0	0	0	2	1	2	1	2	1	1	1	2	1	1	0	2	0	0	0	0	0	0	1	0
OD: 0.5	Independant replicate 1													Independant replicate 2													
Plant	*N. benth*			MM 1			MM 2			MM 3			MM 4			*N. benth*			MM 1			MM 2			MM 3			MM 4			MM 5			MM 6		
Position	x	y	z	x	y	z	x	y	z	x	y	z	x	y	z	x	y	z	x	y	z	x	y	z	x	y	z	x	y	z	x	y	z	x	y	z
Empty	0	0	0	0	2	0	2	0	0	2	0	0	1	1	1	0	0	0	1	2	0	1	2	2	0	2	0	1	1	1	0	1	2	0	2	0
tdt1	0	0	0	3	2	0	0	0	3	0	3	3	3	2	3	0	0	0	3	3	1	2	2	1	0	2	0	3	2	3	2	3	0	3	3	3
Inf1	2	2	3	0	2	0	0	0	0	2	0	0	0	3	1	2	2	2	1	2	2	2	2	2	2	1	1	0	3	1	0	0	2	0	1	0

As the strain Agl1 triggered unspecific responses in tomato, different *A. tumefaciens* strains were tested, including 1D1249, GV3101 and LBA4404. Virus induced delivery was also assessed, with the use of potato virus X (PVX) and tobacco rattle virus (TRV). As an alternative to infiltration, toothpick inoculations (Vleeshouwers at al., 2008) were also tested, using PVX constructs. Out of all the methods tried, the most reproducible results were observed with the agro-infiltration based delivery of PVX via strain GV3101. This expression system gave the best results at an OD_{600}: 0.05 (Figure V.1). The set of negative controls was expanded to include GFP truncated, Inf1 and 11F, a non-functional truncated version of the cell death

inducer CRN2, and did not trigger any response in the infiltrated areas. In contrast, positive controls including cell-death and necrosis inducing Crinklers CRN1 and CRN2 (Torto et al., 2003), as well as a functional truncated version of CRN2 called 10F, reproducibly yielded recognition responses. Indeed, CRN2 triggered a strong cell death response and 10F a weaker but still clearly recognisable cell death. In CRN1 infiltration sites, the weak accumulation of phenolic compounds could be observed under UV light. The constructs GV3101-PVX: CRN2, 10F, 11F and CRN1 were kindly provided by Dr. Sophien Kamoun, with the help of Dr. Edgar Huitema.

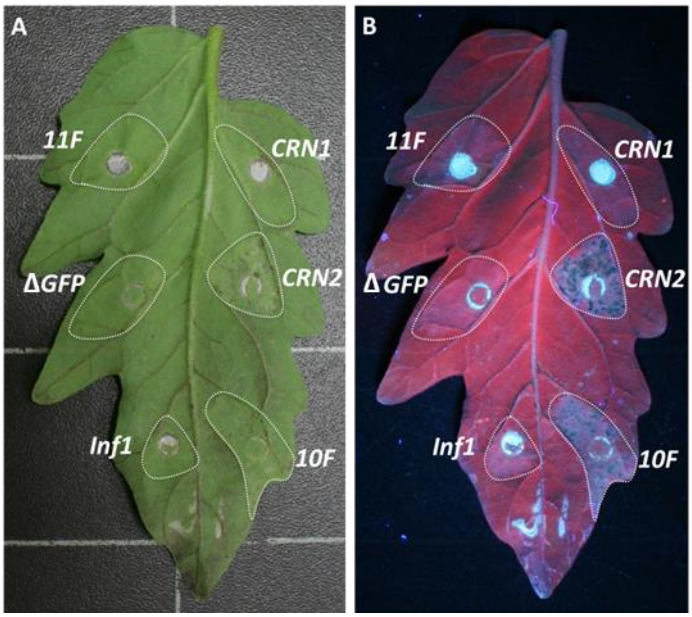

Figure V.1: Agroinfiltration test of leaves from tomato *cv.* Moneymaker with PVX constructs (ΔGFP, Inf1, CRN1, CRN2, 10F and 11F) in *A. tumefaciens* strain GV3101. Each OD_{600} was adjusted to 0.05. Pictures were taken at 7dpi under normal light (A) and UV light (B).

With a lower OD_{600} of 0.01, some of the expected cell deaths were marginally detectable, and overall the HRs were weaker (Figure V.2). In contrast, at a higher OD_{600} of 0.1, some phenolic compounds accumulation and weak HRs were observed within the negative controls (Figure V.2).

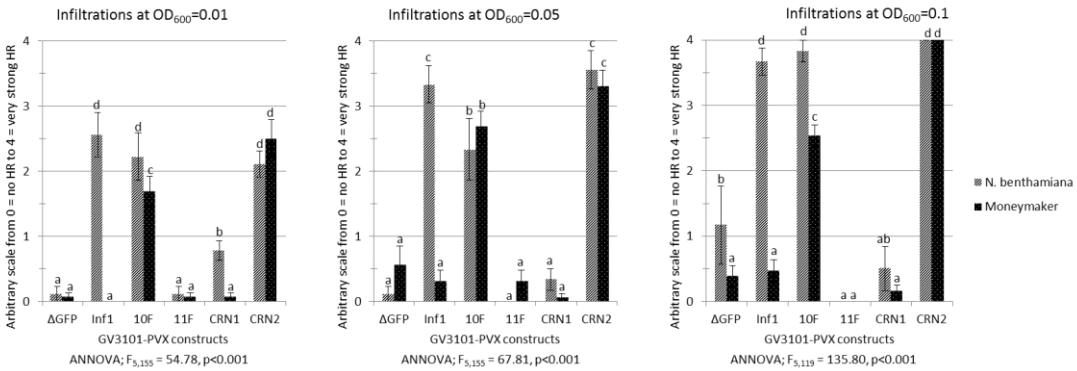

Figure V.2: Graphical representation of HR scores after agro-infiltration of the GV3101-PVX constructs delta GFP, Inf1, 10F, 11F, CRN1 and CRN2 in *N. benthamiana* and the tomato *cv.* Moneymaker at different OD_{600}. Phenotypes are scored 8dpi under UV light, using an arbitrary scale from 0 = no HR to 4 = strong HR. In total, 17 infiltration sites per constructs were scored over two independent replicates for the *cv.* Moneymaker, and nine for *N. benthamiana*. Standard errors are shown in the graphs, as well as the ANNOVA results, with the significances indicated with letters above the histograms.

B. Screening of 96 *P. infestans* RXLR effectors in the tomato association panel, and identification of Avr-Ph3 candidates

In order to identify *Avr-Ph3*, an effector screen was setup in a collection of susceptible, resistant *Rpi-Ph2* and resistant *Rpi-Ph3* tomato lines, forming an association panel. As mentioned before, a suitable delivery system was optimised for tomato, consisting of the *A. bacterium* strain GV3101 combined with PVX. Thus, the library of 96 RXLR effectors available at the JHI in Agl1-pGRAB was re-cloned in that system by Dr Sophie Mantelin (JHI) (Table II.3), and screened in the association panel. Five effectors: PITG_16240, PITG_16427, PITG_23015, PITG_23226 and PITG_11484 triggered highly significant ($p<0.001$) reproducible HRs in the *Rpi-Ph3* tomato lines only, compared to the other lines, and in at least three independent replicates (Figure V.3 and Figure V.4). These results are based on the analysis of at least six infiltration sites, per constructs, per each individual line, were scored over at least three

independent replicates. Results were bulked between *Rpi-Ph2*, *Rpi-Ph3* and susceptible lines, with *N. benthamiana* as a control. In total, at least 43 infiltration sites, per construct and per bulk, was scored, resulting in the identification of PITG_16240, PITG_16427, PITG_23015, PITG_23226 and PITG_11484 as *Avr-Ph3* candidates. Interestingly, although *Rpi-Ph3* is closely related to *Rpi-vnt1* on a sequence level with more than 84% identity (ORFs) on the nucleotide level, Avr-vnt1 (PITG_16294) did not yield any recognition responses in any tomato accessions including the *Rpi-Ph3* resistant tomatoes (Figures V.3 and V.4).

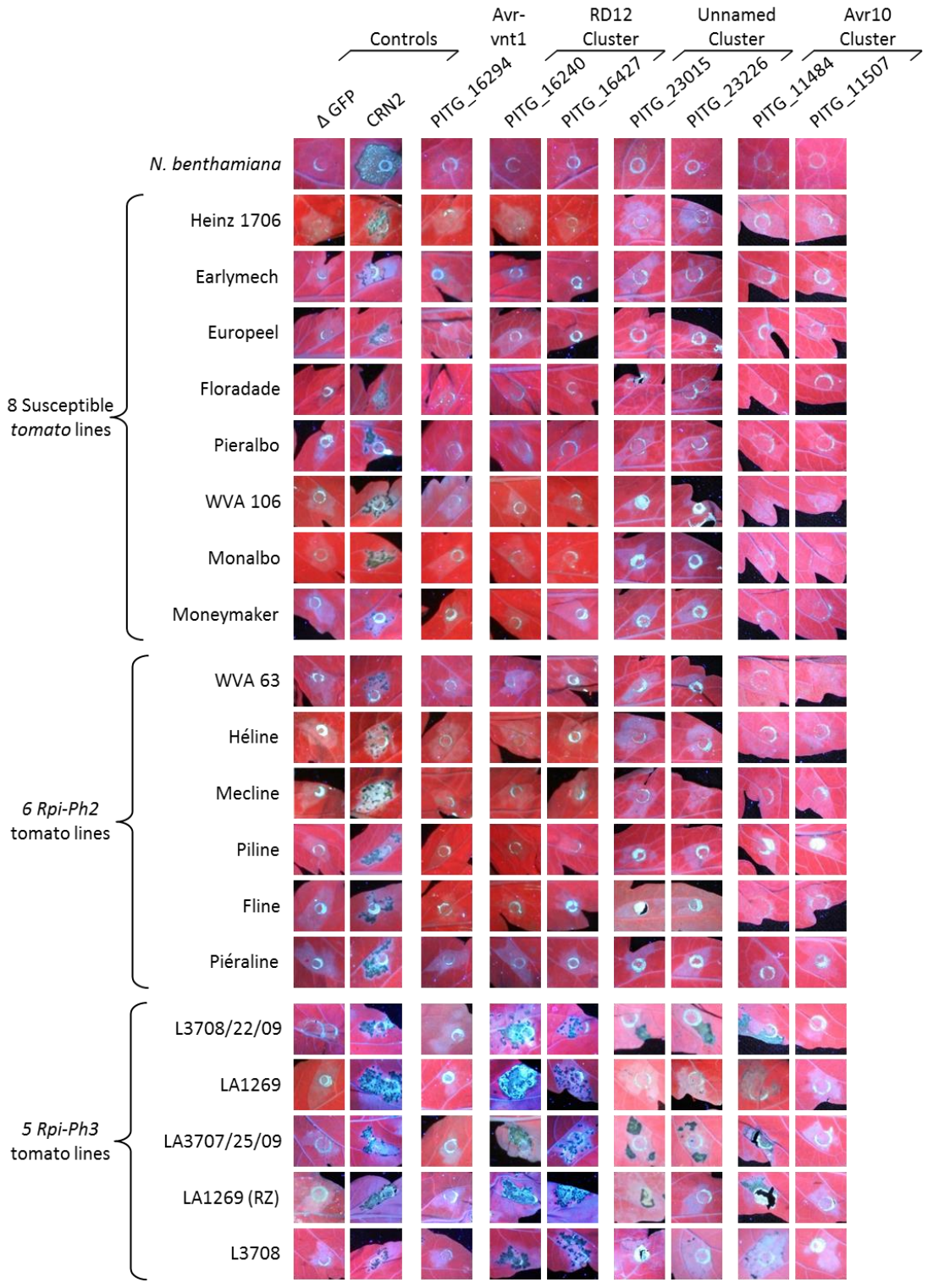

Figure V.3: Five *Avr-Ph3* candidates have been identified in the tomato effector screen: PITG_16240, PITG_16427, PITG_11484, PITG_23015 and PITG_23226. The screen was performed in an association panel of tomatoes containing eight susceptible lines, six *Rpi-Ph2* containing lines and five *Rpi-Ph3* containing lines. An *in silico* analysis

of these effectors shows that they can be classified in three clusters, as indicated above the pictures in the figure V.3. The screening results of *Avr-vnt1* are also shown due to the high similarity found between *Rpi-vnt1* and *Rpi-Ph3* (Zhang et al., 2014). Agro-infiltrations were performed at an OD600=0.05 and results were scored at 8dpi. The cell death inducer CRN2 and the non-functional truncated GFP (ΔGFP) constructs were used as positive and negative control, respectively. All RXLR effectors, cloned into PVX were delivered for transient expression via *A. tumefaciens* strain GV3101. Pictures were taken under UV light.

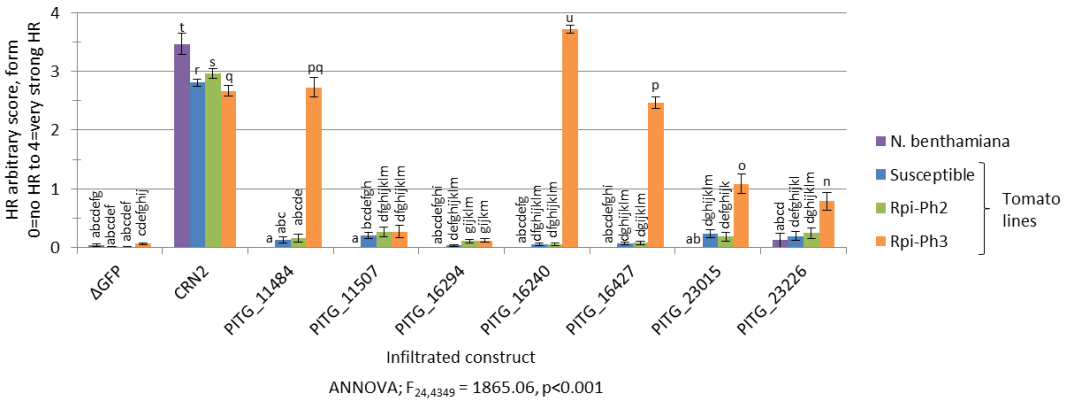

Figure V.4: Graph of the HR scores after agro-infiltration of the GV3101-PVX constructs in *N. benthamiana* and the tomato association panel. Infiltrations were performed at OD_{600}= 0.05. Phenotypes were scored 8dpi under UV light, using an arbitrary scale from 0 = no HR to 4 = strong HR. At least six infiltration sites, per constructs, per each individual line, were scored over at least three independent replicates. Results were bulked between *Rpi-Ph2*, *Rpi-Ph3* and susceptible lines, with *N. benthamiana* as a control. In total, at least 43 infiltration sites, per construct and per bulk, was scored. Standard errors are shown in the graphs as well as the ANNOVA results, with the significances indicated with letters above the histograms. The different tomato lines are colour coded, with the susceptible lines in blue, the *Rpi-Ph2* lines in green and the *Rpi-Ph3* lines in orange.

C. *In silico* analysis of the *Avr-Ph3* candidates identified

The five *Avr-Ph3* candidates identified above were analysed *in silico*, to establish their phylogenetic relationship. Indeed, a MCL cluster analysis revealed that these candidates can be classified into three families based on their sequence similarities (Figure V.5) (Gaëtan Thilliez's personal communication, JHI). PITG_16240 and PITG_16427 are part of the RD12 cluster containing six members (Figures V.5 A and B; top panel). PITG_23015 and PITG_23226 are part of a smaller cluster that contains three members (Figures V.5 A and B; middle panel). Finally, PITG_11484, also known as Avr10, only shares sequence identity with one truncated RXLR (Figures V.5 A and B; bottom panel).

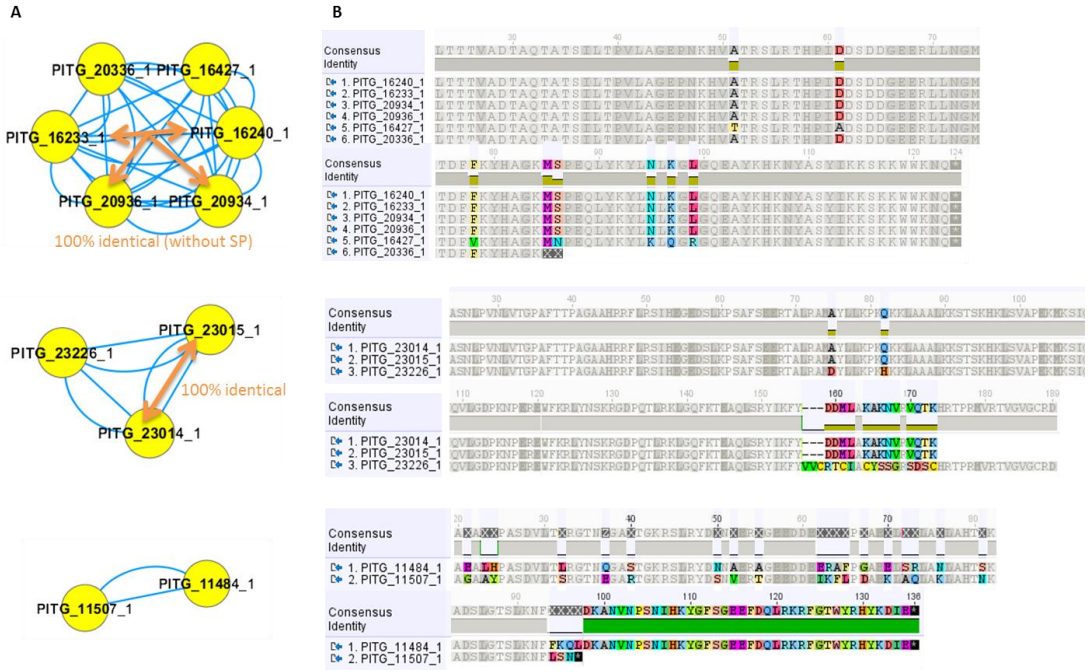

Figure V.5: Avr-Ph3 candidate effectors represented within their respective clusters. A) Schematic of the different members of a given cluster (Gaëtan Thilliez personal communication, JHI). Clusters were built using a BLASTp analysis of the effectors (without signal peptides), with a maximum e-value of 1e-5. Effectors were then grouped using the MCL algorithm, with an inflation value of 6. Cytoscape v2.8.3 was used for visualisation purposes. The blue lines illustrate when two protein sequences share significant sequence similarity. The orange arrows show when two proteins share 100% sequence homology. B) Alignment of the different members of a given cluster in Geneious. Non-synonymous SNPs that result in amino acid changes are highlighted in bright colour, while the conserved amino acid sequences are shown in grey.

2. Confirmation of the *Avr-Ph3* candidates

A. Co-infiltration of the *Avr-Ph3* candidates with *Rpi-Ph3* in *N. benthamiana*

Rpi-Ph3 was cloned (Zhang et al, 2014) whilst the screening for *Avr-Ph3* was conducted. Thus, co-infiltrations in *N. benthamiana* became possible between *Rpi-Ph3* and the candidates identified above. Furthermore, to also ensure that no candidates have been missed due to

ascertainment problems in tomato, all 96 RXLR effectors already available in the Agl1-pGRAB set were tested via co-infiltrations with *Rpi-Ph3* in *N. benthamiana*. The cloning of PITG_16240 in Agl1_pGRAB system was unsuccessful. However, PITG_16240 and PITG_16427 are so similar on the sequence level that they were considered as redundant in this experiment. Interestingly, only the co-infiltrations of *Rpi-Ph3* with the previously identified *Avr-Ph3* candidates PITG_23015 and PITG_23226 yielded reproducible HRs (Figure V.6). This suggests that PITG_23015 and PITG_23226 are both recognised by *Rpi-Ph3*, confirming that they are two potential variants of *Avr-Ph3* (Figure V.6). As expected, the negative control PITG_16294 (*Avr-vnt1*) is not recognised by *Rpi-Ph3*. Similarly, candidates PITG_16427, , PITG_11507 and PITG_11484 do not elicit a *Rpi-Ph3* specific recognition response (Figure V.6). This suggests that PITG_16427 (PITG_16240 by extension) and PITG_11484 recognition in the *Rpi-Ph3* tomato lines utilised within the association panel may not be the result of *Rpi-Ph3* based recognition but potentially the response to another, unknown *R* gene. However, another interpretation could be that the recognition of the aforementioned effectors may rely on components of the *Rpi-Ph3* signalling machinery that are evolutionary divergent between Tomato and *Nicotiana benthamiana*. Overall, the observations are based on five independent replicates, with between two to six infiltration site per construct.

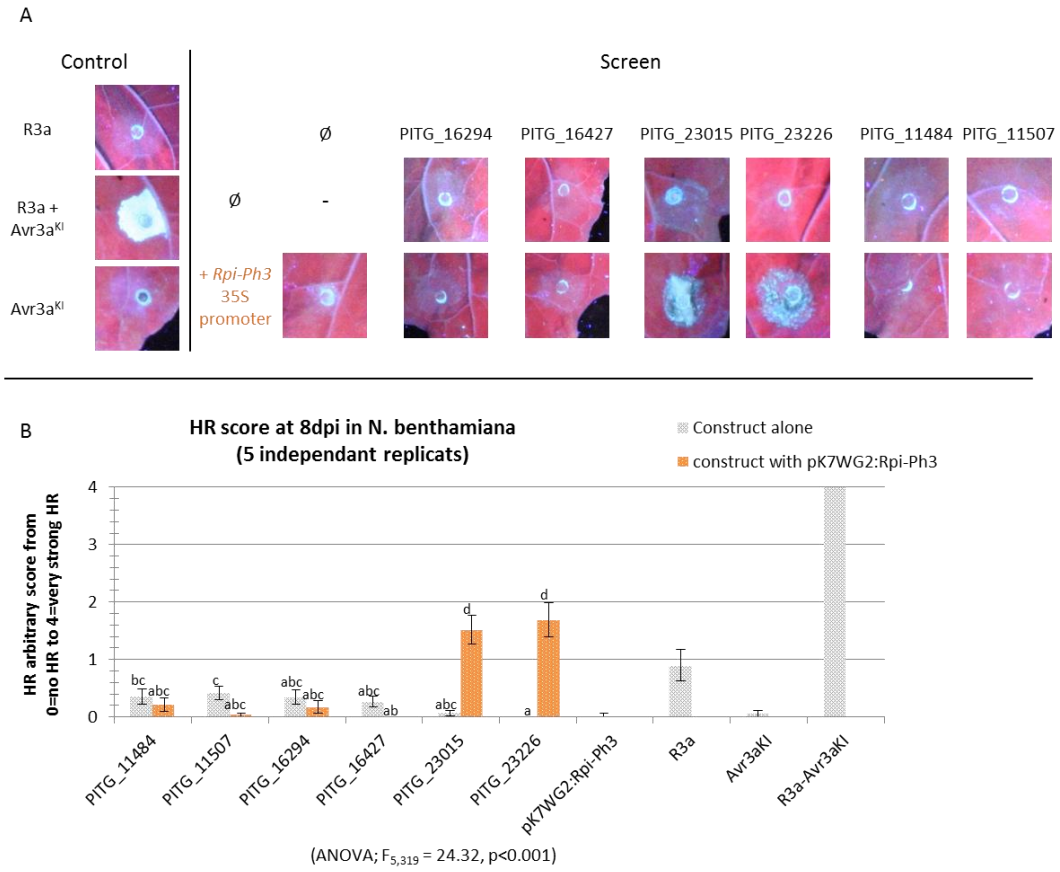

Figure V.6: Co-infiltration of *Rpi-Ph3* and the *Avr-Ph3* candidates in *N. benthamiana*. PITG_16240 is absent of this experiment as its cloning into the pGRAB vector was unsuccessful. Being so closely related to PITG_16427, the two effectors were considered as redundant. The well-established R3a and Avr3a^{KI} recognition was used as control (Left hand side of panel A). Co-infiltrations were performed at OD_{600}=0.5 at a 1:1 ratio and with P19 at a final OD_{600} of 0.01. Results were scored at 8dpi. A) Pictures of the infiltration sites under UV light. The effectors were infiltrated on their own (Right hand side of panel A, first line) and the effectors co-infiltrated with *Rpi-Ph3* (Right hand side of panel A, the second line). B) Graph representing the phenotypic response observed in five independent replicates.

Western blots were performed to ensure stability of the constructs PITG_11484, PITG_11407, PITG_16240, PITG_16427, PITG_23015 and PITG_23226 in *N. benthamiana* (Figure V.7). The empty control tagged with GFP was also tested in the western blot. Two

different protocols were used for the protein extraction and western blot analysis; including a fast diagnostic method based on a quick protein extraction directly in the loading buffer; and a more rigorous but time consuming method based on a GTEN protein extraction buffer, in which samples are cleaned before adding the loading buffer (Chapter II, Part IV.2). Bands of the expected sizes were observed for each construct (Figure V.7). However, bands for PITG_23015 and PITG_23226 were much weaker if compared to the other constructs using the diagnostic method (Figure V.7A) and completely absent with the traditional GTEN method (Figure V.7B).

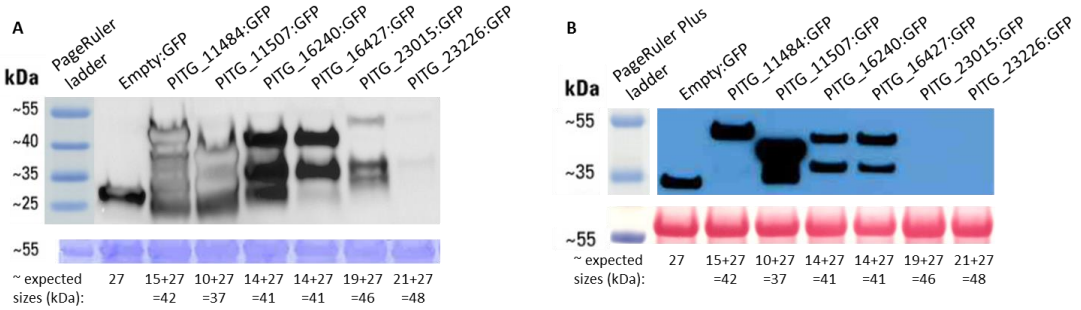

Figure V.7: Western Blots of the different *Avr-Ph3* candidates: PITG_11484 (and related PITG_11507), PITG_16240, PITG_16427, PITG_23015 and PITG_23226, together with the negative empty control. A) Western blot obtained with the quick diagnostic method, based on the extraction directly in the loading buffer. B) Western blot obtained with the traditional GTEN protein extraction. The loading of the samples was checked with (A) Coomassie blue and (B) Ponceau red. Different ladders from ThermoFisher were used: (A) PageRuler Prestained Protein Ladder and (B) the PageRuler Plus Prestained Protein Ladder. The band sizes are indicated. The immunodetection was realised with a rabbit GFP primary antibody and an anti-rabbit secondary antibody. Pictures were taken (A) directly of the membrane using a G:Box (from Syngene) or (B) after a film was exposed to the western blot.

B. Boost of pathogenicity assay with the *Avr-Ph3* candidates

The different *Avr-Ph3* candidates were tested for their effect on *P. infestans* pathogenicity in *N. benthamiana*. *Avr-Ph3* candidates were transiently expressed in *N. benthamiana* at an OD_{600} of 0.1 with 0.05 of P19. The inoculated regions were subsequently challenged with *P. infestans* isolate 88069-tdT the following day. Infection lesions were measured after seven days post infection and the data of three independent replicates are shown in Figure V.8. PITG_23015 and PITG_23226 gave a highly significant ($p<0.001$) boost to the *P. infestans* growth, compared to the empty control (Figure V.8). Indeed, late blight lesions were more than three times the size if compared to the empty control (Figure V.8). This boost of pathogenicity was even more significant than that observed for the positive control PITG_04097, which was already known to increase *P. infestans* virulence (Dr. Hazel McLellan personal communication). Avr-vnt1 (PITG_16294) did not highly significantly ($p<0.001$) affect the pathogenicity of *P. infestans* compared to the empty control (Figure V.8). PITG_16427, PITG_11484 and PITG_11507 yielded late blight lesions that were slightly, but highly significantly ($p>0.001$), bigger than the lesions observed for the empty controls (Figure V.8-B).

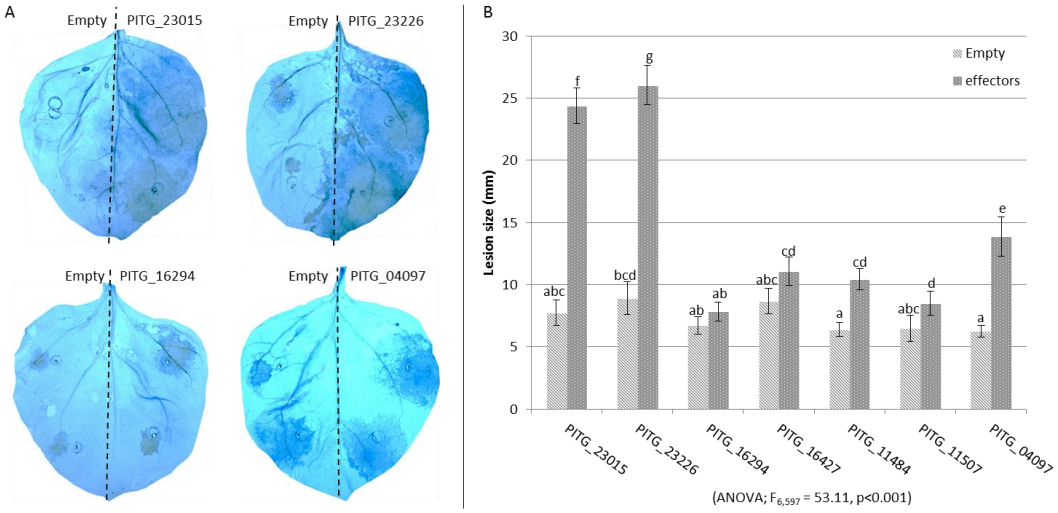

Figure V.8: PITG_23226 and PITG_23015 give a boost of pathogenicity to *P. infestans*. A) Images of *N. benthamiana* leaves infiltrated with *Avr-Ph3* candidates PITG_23015 and PITG_23226, Avr-vnt1 (PITG_16294) and positive control PITG_04097 in parallel to empty vector control; which were inoculated with *P. infestans* isolate 88069-tdT the following day. Leaves were stained after 8dpi with trypan blue, to visualise *P. infestans* growth (in blue) through the leaves. B) Comparison of *P. infestans* lesion sizes, following infiltration of *N. benthamiana* leaves with candidate Avr-Ph3 effectors, PITG_23015, PITG_23226, other effectors recognised in Rpi-Ph3 lines PITG_16427 and PITG_11484, Avr-vnt1 (PITG_16294) and positive control PITG_04097; alongside empty vector. In total, three independent replicates, involving at least 10 inoculation sites per construct per replicate, were studied at 8dpi. An ANOVA ($F_{6,597} = 53.11$, $p<0.001$) was performed on the results and the letters above each histogram highlight significant differences.

III. Discussion

In this study, we assumed that recognition of RXLR effectors underpins the resistance mediated by *Rpi-Ph3*. However, this is solely based on extrapolating the current observation that all *P. infestans Avr* genes identified so far encode RXLR-type effectors (Vleeshouwers et al., 2008; Hein et al., 2009). A screening of already cloned, expressed and relatively

conserved RXLR genes (in Agl1-pGRAB) was conducted to verify this hypothesis. However, preliminary data generated in this chapter has shown that agro-infiltration based screening is more difficult in tomato than it is in potato (Table V.1), and even more difficult than in our model *Solanaceae N. benthamiana*. Nevertheless, *A. tumefaciens*-based transformation of tomato lines has been reported in the literature (Sharma et al, 2009; Islam et al, 2010) and transient delivery of effectors via this system was investigated. After numerous trials of different expression system, I could establish that GV3101-PVX is a suitable transient expression system for the tomato accessions utilised (Figure V.1 and 2). By using the appropriate positive and negative controls, I could demonstrate that there is no impact on the recognition results by using a virus that can move systemically through a plant.

Five effectors gave reproducible HRs in the *Rpi-Ph3* lines only (Figure V.3): PITG_11484, PITG_16240, PITG_16427, PITG_23015 and PITG_23226. However, only two of them, PITG_23015 and PITG_23226, gave reproducible HRs when co-infiltrated with *Rpi-Ph3* in *N. benthamiana*. Importantly, both effectors are phylogenetically related (Figure V.5), which provides further evidence that their recognition by Rpi-Ph3 is specific. It has been shown that other Avr genes from *P. infestans* such as Avr2, Avr3a, Avrblb1 and Avrblb2 (Gilroy et al., 2001; Armstrong et al., 2005; Vleeshouwers et al., 2008; Champouret et al., 2009; Oh et al., 2009) contain closely related family members. To overcome the resistance genes contained in their hosts, oomycetes utilise various strategies, which are illustrated by different examples in the literature. Some effectors such as Avr3a evade recognition through diversification (Armstrong et al., 2005). Indeed, $Avr3a^{KI}$ is the recognised form by R3a, but some *P. infestans* developed an $Avr3a^{EM}$ unrecognised form, which avoids recognition (Armstrong et al. 2005). Other effectors, like Avr4 or Avr10, are lost from the genome of *P. infestans* isolates capable of overcoming *R4* and *R10*, respectively (van Poppel et al., 2008; Jiang et al., 2006). Finally, a last example is found with *Avr2,* in which expression is lost in virulent *P. infestans* isolates in *R2* plants (Gilroy et al., 2011).

According to Dr. John W Scott, a tomato breeder who was associated with the USDA funding that supported my PhD study, *Rpi-Ph3* provides very durable resistance towards the typical US isolates that infect susceptible lines. It would therefore be important to assess the diversity of the *Avr-Ph3* family in international isolate and to then assess the recognition of these variants by Rpi-Ph3. Furthermore, a heterologous expression system could be utilised to demonstrate that recognition of Avr-Ph3 in tomato lines carrying *Rpi-Ph3* yields resistance. This could, for example, be achieved by using *Pseudomonas syringae* pv. tomato DC3000 (Xin et al., 2013).

It was interesting to note that PITG_11484 and closely related effectors PITG_16240 and PITG_16427 triggered HRs in the *Rpi-Ph3* tomato lines (Figure V.3), but did not yield an HR upon co-infiltration with *Rpi-Ph3* in *N. benthamiana* (Figure V.5). The correct expression of the different effectors in *N. benthamiana* were confirmed by western blot (Figure V.6) and showed that the non-recognised effectors were stable upon transient expression. I therefore conclude that PITG_11484, PITG_16240 and PITG_16240 are not Avr-Ph3, and could be recognised by another *R* gene(s) present in all the *Rpi-Ph3* lines. Indeed, Rpi-Ph3 resides on the lower end of chromosome 9, which has been shown to be a NB-LRR rich region in potato (Jupe et al., 2013). In addition to *Rpi-Ph3*, functional genes effective towards late blight in this locus include *Rpi-vnt1* (Pel et al., 2009; Foster et al., 2009), *R8*, *R9a* (Jack Vossen personal communication) and *Rpi-ver1* (Ingo Hein personal communication, Wageningen University). Based on the high similarity between the potato and the tomato genomes, it is feasible that another, functional *R* gene co-resides within the locus that harbours *Rpi-Ph3* in the association panel. This could explain, for example, how several *R* genes have been introgressed at the same time as *Rpi-Ph3* in different breeding efforts to produce *Rpi-Ph3* resistant lines. Indeed, as mentioned in chapter III, there was no breeding marker closely associated with *Rpi-Ph3* until recently (Figures III.4 and III.5). The breeding markers used until then were, however, covering a wider region of the bottom part of chromosome 9. However,

it is important to acknowledge that the recognition of the PITG_16240, PITG_16427 and PITG_11484 in the *Rpi-Ph3* tomato lines could rely on components of the *Rpi-Ph3* signalling machinery that are evolutionary divergent between tomato and *Nicotiana benthamiana*. This would explain the absence of recognition in the co-infiltration assays but wouldn't rule out those effectors as Avr-Ph3 candidates.

The tomato association panel used in this study contained eight susceptible lines, five *Rpi-Ph3* resistant lines and seven *Rpi-Ph2* resistant lines. Out of the 96 effectors screened, no effector was consistently recognised in the susceptible lines and, surprisingly, no effector was identified that would explain *Rpi-Ph2* resistance. This suggests that *Avr-Ph2* is not part of the core RXLR set of effectors used in this study, and might even not be an RXLR all together. It is conceivable, for example, that Avr-Ph2 could be an aploplastic effector instead. That is the case for the *Cladosporium fulvum* Avr2 effector, which is secreted in the tomato apoplast upon infection (Luderer et al., 2002; Rooney et al., 2005; Song et al., 2008). This would be very interesting to see as it would be the first apoplastic *P. infestans* avirulence protein reported (Armstrong et al., 2005; Hein et al., 2009; Raffaele et al., 2010; Cooke et al., 2012). Nevertheless, unpublished data by Gaëtan Thilliez suggests that the reference genome of *P. infestans* T30-4 might contain more than 1500 putative RXLR effectors, which is significantly higher than the 425 RXLR effectors predicted by Whisson et al (2007), or the 563 reported RXLR effectors by Haas et al. (2009). Therefore, many additional RXLR effectors might need cloning and screening. Alternatively, Avr-Ph2 could be identified through a genetic screen.

Chapter VI: General Discussion and Future Work

I. General Discussion

The biggest threat to potato and tomato productions worldwide is late blight disease caused by the oomycete pathogen *Phytophthora infestans*. Wild *Solanaceae* species are able to withstand multiple biotic and abiotic stresses, suggesting that many unexplored natural sources of resistance exist for utilisation in breeding programs. Traditional breeding methods have failed to effectively protect crops from pathogens, due to the inherent difficulties in isolating new resistances and then deploying them in a timely fashion. The deployment of *Rpi-blb1* and *Rpi-blb2* from the diploid potato *S. bulbocastanum,* for example, was time costly, mainly due to crossing barriers with the cultivated tetraploid potato: *S. tuberosum* (Hanneman, 1999). It took 47 years of bridge crosses to successfully introgress *Rpi-blb2* into the potato *cv.* Bionica and Toluca (Haverkort et al., 2009). Similarly, complicated breeding techniques such as somatic hybridisation and bridge crosses were required to introgress *Rpi-blb1* into cultivated potato (Hermsen and Ramanna, 1973; Helgson et al., 1998), methods which require several years to complete. In total, more than 40 years of traditional breeding efforts were required to introduce a *Rpi-blb1* resistant cultivar to the market (van der Vossen et al., 2003). However, the discovery of functional *Rpi-blb1* orthologs *Rpi-sto1* from *S. stoloniferum* and *Rpi-pta1* from *S. papita* (Vleeshouwers et al., 2008; Wang et al., 2008), made breeding for this resistance much more accessible. Indeed, direct crossing between *S. stoloniferum* and *S. tuberosum* is possible (Vleeshouwers et al., 2008) and *Rpi-pta1* is currently being used for classic introgression into cultivated potato (Vleeshouwers and Oliver, 2014). However, the development of resistant cultivars resulting from direct crossings still requires about 10 years+ of breeding, as numerous generations of back-crosses and

selections are necessary (Jacobsen and Hutten, 2006). Since the turn of the millennium, multiple resistant to *P. infestans* genes (*Rpi genes*) have been cloned using traditional genetic approaches (Chapter I, table I.2). This enabled another step forward in the generation of resistant cultivars by utilising GMO approaches (Jones et al., 2014).

The introduction of modern sequencing technologies into crop research has had a major impact on fundamental and applied research. Sequencing costs followed for a long-time Moore's law that described a trend by which the price for computational power halved approximately every two years as the computational power doubled. However, since 2007 sequencing costs have seen an unprecedented fall. This is best illustrated by the price of sequencing a human genome, which is estimated to have cost $10 million in 2007 and has been reduced to about $1000 by 2014 (Hayden, 2014). Indeed, with the recent advances in genome sequencing technologies, entire crop genomes are now available. For example, eleven years since sequencing the model plant *Arabidopsis thaliana*, the genomes of two important *Solanaceae* crop plants, potato and tomato, were reported (PGSC, 2011; TGC, 2012).

The further development of targeted sequencing that significantly reduces the genome complexity, and detailed analysis and re-annotation of these genomes has accelerated the identification of functional *R* genes. For example, 755 NB-LRR genes have been identified in the sequenced S*olanum tuberosum* group Phureja clone DM1-3 516 R44 (DM), and their phylogenetic relationships as well as their physical locations has been described on the 12 potato chromosomes (Jupe et al., 2012; 2013). Similar work was performed for the *S. lycopersicum* linage Heinz 1706 and 397 NB-LRR genes were annotated on the 12 tomato chromosomes (Andolfo et al. 2014). These studies formed the basis of a novel *R* gene enrichment and sequencing platform (RenSeq) that enables the improved annotation of resistance genes in sequenced genomes and facilitates rapid mapping and cloning of

resistances via bulked-segregant analysis (BSA) (Jupe et al., 2013). However, the use of RenSeq is not limited to NB-LRR annotations and mapping. Indeed, in this thesis, a new tool has been developed from RenSeq technology that enables screening of germplasm collections and wild *Solanaceae* plants for novel resistances. The technology, referred to as dRenSeq, is based on the stringent mapping of RenSeq-derived reads against a reference set that includes characterised, functional NB-LRRs. Importantly, the reference list is versatile and can include late blight resistance genes, genes effective against nematodes, viruses and other pests and pathogens and can be updated at any stage (Chapter IV; Van Weymers et al., 2016). As illustrated for *Rpi-vnt1*, dRenSeq can be used to ascertain if germplasm accessions contain already known resistances or a novel source to control pathogens. In the latter case, RenSeq reads used for dRenSeq can be utilised in a bulked-segregant analysis as detailed by Jupe et al. (2013) and in Chapter III. Indeed, the mapping of a resistance from the potato segregating population B3C1HP using BSA RenSeq analysis has been described in the course of this thesis (Chapter III).

Other variations of RenSeq enabled the accelerated cloning of new *Rpi* genes, as detailed for the identification of *Rpi-amr3i*, from *Solanum americanum* (Witek and Jupe et al., 2016). In the study by Witek and Jupe et al., (2016) RenSeq was used in conjunction with long read Single-Molecule Real-Time (SMRT) sequencing, which allowed the *de novo* assembly of entire NB-LRRs (Witek and Jupe et al., 2016). Indeed, initial RenSeq was based on Illumina short read sequencing (Jupe et al., 2013), and can prove challanging when the re-assembly of entire NB-LRRs is required. Indeed, based on the evolution of this large family, which often involved gene duplications followed by diversification, high sequence identity can exist between paralogs and alleles (Meyers et al., 1999). This problem can be acerbated when investigating species more distantly related to the sequenced reference genome.

A further variation of RenSeq for the faster cloning of disease-resistance genes was made by combining RenSeq with mutagenesis and exome capture (Steuernagel and Periyannan et al., 2016). This method called MutRenSeq, permits the identification of resistance genes without previous fine mapping and enables studies on plants species for which whole genome sequencing is not suitable or available (Steuernagel and Periyannan et al., 2016). In their study, Steuernagel and Periyannan et al. (2016) reported the cloning of two *Puccinia graminis* resistance genes, Sr22 and Sr45, from hexaploid wheat.

In Figure VI.1, an overview of the current advances in resistance studies after the development of Next Generation Sequencing (NGS) technologies and target enrichment is illustrated, and will be discussed further below.

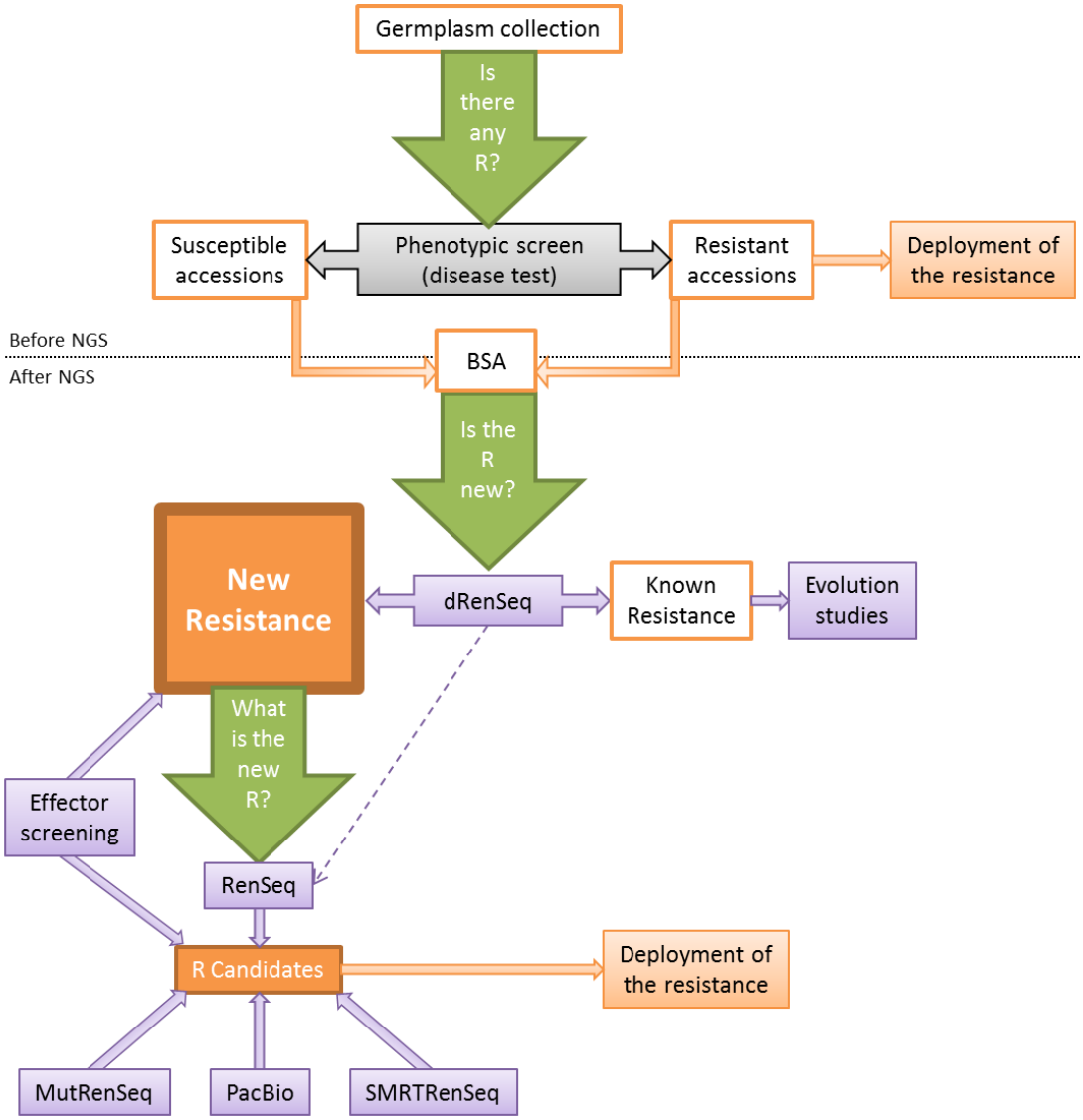

Figure VI.1: Pipeline for the discovery of new resistances in the era of 'omics' technologies. The Figure illustrates the different methods developed in recent years (including this thesis) to identify new resistance genes. The green arrows describe the biological questions which are driving the studies (with R abbreviating resistance). Shown in purple are the methods directly derived from the advances made in Next Generation Sequencing (NGS) technology. The dotted black line emphasises how NGS has revolutionised the study and deployment of resistance genes in the post-genomic era.

Pivotal for the success of RenSeq is sufficient diversity within the bait library to enable capture of unrelated NB-LRRs from the plant accessions studied. Previous studies by Jupe et

al., (2013) demonstrated experimentally that approximately 80% sequence homology between the 120nt RNA baits and the target sequences is sufficient to facilitate enrichment. Sequencing genomes of related plant species provides an opportunity to analyse probe library efficacy in retrospect and, if required, to include new genes in the library design. The rational for re-designing the RenSeq probe library following the analysis by Jupe et al., (2013) and Andolfo et al., (2014), was to ensure that the new library would successfully enrich for sequences related to the newly identified NB-LRRs in other *Solanaceae* species (Chapter III).

For resistances to be most effective, it is critical to understand the mechanisms of defence. Indeed, this knowledge is essential to predict the potential durability of resistances deployed (Birch et al., 2008). For example, if a field contains known resistances, and if surrounding outbreaks of *P. infestans* can be monitored for their virulence spectrum, a farmer could make an educated choice whether or not to spray fungicides in his field. Haverkort et al. (2016) showed in their study that fungicide use could indeed be reduced by 80%, if multiple effective *R* genes are deployed in a spatial-temporal monitored manner. To facilitate this, the knowledge gained for crops has also been mirrored for the respective pathogens. The *P. infestans* genome was published in 2009 by Haas et al., and enabled the genome-wide cataloguing of RXLR-type effector candidates.

To date, all *P. infestans* avirulence genes (effectors that are recognised and consequently trigger incompatibility) contain a canonical RXLR domain (Armstrong et al., 2005; Hein et al., 2009; Raffaele et al., 2010; Cooke et al., 2012) and 563 putative RXLR effectors have been described in the T30-4 reference genome (Haas et al., 2009). However, unpublished data by Gaëtan Thilliez suggest that this number could increase to over 1500 putative RXLR effectors, highlighting the vast complexity of potential new virulent forms.

On a molecular level, a detailed understanding of effector and *Avr* gene diversity provides a valuable tool for the deployment and monitoring of newly identified and already deployed

resistances (Birch et al., 2008; Chapter V). Indeed, *P. infestans* has been reported as highly adaptable, being able to overcome resistance within few years in the field. A well-known example is the Scottish *cv.* Pentland Dell, which contains *R1*, *R2* and *R3* from the wild potato species *S. demissum*, and which resistances were defeated within a short number of growing seasons (Hein et al., 2009b). By studying the cognate effectors various evolutionary processes have been discovered that explain virulence on potatoes with characterised resistances. As mentioned in Chapter V, *P. infestans* has different strategies to overcome resistances. Indeed, it is able to lose redundant effectors from its genome, as seen with the absence of Avr4 from isolates overcoming the R4 resistance for example. The expression of some *Avr* genes can also be discontinued in some *P. infestans* isolates, as it is the case for Avr2 (Gilroy et al., 2011). Effector diversification, on a sequence level, has also been observed in late blight isolates able to overcome a resistance. A well-documented example of this strategy is Avr3a^{KI}, which triggers resistance upon recognition by R3a (Armstrong et al. 2005). A slightly different form of this effector, Avr3a^{EM}, has been identified in different *P. infestans* isolates and is able to avoid the recognition by R3a (Armstrong et al. 2005; Cardenas et al., 2011). Recent work took advantage of this knowledge and was carried out to artificially enhance the spectrum of R3a recognition to enable the recognition of both Avr3a^{KI} and Avr3a^{EM} (Segretin et al., 2014; Chapman and Stevens et al., 2014). Interestingly, although enhanced recognition was achieved, resistance towards *P. infestans* isolates carrying Avr3a^{EM} remains elusive, which suggests that recognition and resistances are not necessarily coupled (Chapman and Stevens et al., 2014).

Effectors are also used to identify new resistance, or to identify resistances that display the same recognition specificity. Stacking resistances has shown to provide more durability compared to the deployment of single *Rpi* genes (Jo et al., 2014). As mentioned above, it is paramount that combined resistances are complementary, rather than functionally redundant, and effectors allow this discrimination. Use of effectoromics (Vleeshouwers et

al., 2008 and 2011; Vleeshouwers and Oliver; 2014), resulted in the identification of numerous *Rpi* genes with similar recognition specificity in potato, such as *Rpi-blb1* from *S. bulbocastanum*, *Rpi-pta1* from *S. papita* and *Rpi-sto1* from *S. stoloniferum* (Vleeshouwers et al., 2008).

In Chapter V, effectoromics has been used in a slightly different manner. Instead of using known Avr effectors to identify the corresponding *R* genes as described in Vleeshouwers et al. (2008), an established association panel for *Rpi-Ph3* was screened with an array of RXLR effectors to identify Avr-Ph3. In tomato, effective resistances to *P. infestans* are limited, and breeders mainly rely on *Rpi-Ph2* and/or *Rpi-Ph3* genes (Foolad et al., 2008). Located on chromosome 10 and 9, respectively (Moreau et al., 1998, Chunwongse et al., 2002, Zhang et al., 2013 and 2014), these genes have been introgressed into several commercial tomato cultivars (Foolad et al., 2008) and provide effective resistance towards many US isolates of *P. infestans*. Little was known about these two genes when my PhD study commenced. However, significant advances were made on *Rpi-Ph3* with its fine mapping reported in 2013 (Zhang et al., 2013) and the gene was then cloned a year later (Zhang et al., 2014). Nevertheless, the cognate *P. infestans* effector that triggers the plant resistance upon perception by *Rpi-Ph3* remained elusive. Through the screening of the *Rpi-Ph2* and *Rpi-Ph3* association panels, two putative *Avr-Ph3* candidates from the same phylogenetic cluster, PITG_23015 and PITG_23226, were identified (Chapter V). The highly similar effectors triggered a recognition response specifically in the *Rpi-Ph3* association panel and not *in Rpi-Ph2* containing plants or susceptible tomato accession. Other effectors, PITG_16240, PITG_16427 and PITG_11484 also elicited a response in the association panel, but did not yield specific recognition responses upon co-infiltration with *Rpi-Ph3* in the model *Solanaceae* plant *N. benthamiana* (Chapter V). A limited study suggests that PITG_23015 and PITG_23014 (similar on the sequence level) would be truncated in some late blight isolates, while PITG_23226 would be missing in *P. infestans* isolates able to overcome *Rpi-Ph3* (Gaëtan

Thilliez and Howard Judelson personal communications). This would suggest PITG_23226 is the actual avirulence, whereas the recognition of PITG_23014/23015 can be suppressed, and would follow the same pattern as for the Avr2 family members mentioned before. However, these are preliminary data which remain to be investigated further.

Despite successfully identifying Avr-Ph3 candidates, this study has also highlighted some of the limitations of effectoromics. Indeed, no *Avr-Ph2* candidates were identified within the set of cloned RXLR effectors utilised in the screening. This suggests that *Avr-Ph2* could be an effector within the newly identified 1500+ RXLR effectors, or that this avirulence gene does not encode for an RXLR containing protein. Alternatively, it is also conceivable that *Avr-Ph2* could be a non-proteinaceous avirulence determinant. A further limitation of the effector screen in this study was the identification of a suitable transient expression system (Chapter V). Indeed, unspecific PTI and/or ETI responses against *Agrobacterium tumefaciens* and /or PVX can restrict the range of plants that can be screened for effector recognitions. The implication are that multiple effector expression systems have to be established first and then tested in various plant accessions of any given species. The Agro/PVX system utilised in Chapter V did produce reproducible results but required many independent tests and visualisation under UV light, to discriminate the relatively weak phenotypic responses from background noise, for some of the effectors tested such as PITG_23015 and PITG_23226. This resulted in a low throughput effector recognition assay and, in my experience, I would use effectoromics only on resistant plant accessions after a phenotypic screen has been concluded (Figure VI.1).

II. Future Work

- In Chapter III, I have designed a RenSeq bait library to encompass the latest NB-LRR gene models from the *Solanaceae* species potato and tomato. RenSeq has proven very useful as a tool in the study of resistances. Since the library design, new data

have been generated, for *Rpi* genes in other *Solanaceae* species such as *N. sylvestris* and pepper. An *in silico* analysis could be conducted to assess the appropriate representation of baits towards the newly identified NB-LRRs and, if required, update probe libraries accordingly.

- Furthermore, the design of different probe libraries to address individual biological question could be considered. In the case of dRenSeq, for example (Chapter IV), the design of probes specific for known, functional *R* genes only could be established. This would make the probe library much smaller and reduce the genome complexity even further. This, in turn, would make dRenSeq even more affordable, and would develop the diagnostic potential fully, as many different plant accessions could be studied simultaneously. As shown in Chapter IV, many resistant CPC accessions have been identified that could be screened with this dRenSeq bespoke library.

- In chapter V, PITG_23015 and PITG_23226 have been identified as *Avr-Ph3* candidates. The next step would be to test these effectors in a gain of avirulence study. This could involve a heterologous expression system in tomato such as delivery of *Avr-Ph3* through the type III secretion system of *Pseudomonas syringae* pv. *tomato* DC3000 (Pst).

- Furthermore, a comprehensive expression study of PITG_23015 and PITG_23226 in diverse isolates of *P. infestans* could also be established, and would give valuable information for the monitoring of *Rpi-Ph3* resistance in the field. Indeed, it would provide insights into the frequency of *P. infestans* isolates that could potentially evade this resistance. Newly identified variance of the Avr-Ph3 candidates would

then require testing for recognition upon co-infiltration with *Rpi-Ph3* and test of avirulence through the heterologous Pst system.

- The screen for Avr-Ph3 in Chapter V identified another couple of related effectors, PITG_16240 and PITG_16427, which were consistently and specifically recognised in all the *Rpi-Ph3* lines of the association panel. However, as these effectors did not produce a specific recognition response upon co-infiltration with *Rpi-Ph3*, it is possible that at least one further closely linked gene co-segregates with *Rpi-Ph3* in the association panel. A RenSeq BSA analysis could be carried out on bulks that are specifically selected for their opposing responses towards these additional effectors.

- A similar BSA analysis could also be initiated for the additional resistance identified in *S. okadae* accessions that functions independently of Rpi-vnt1.1 (Chapter IV).

- As discussed previously, there might be a major increase in the number of predicted RXLR effectors in *P. infestans*. Additional information such as MCL cluster analysis, expression, diversity and conservation in different isolates, is required for these effectors. Effectors that are expressed might also be cloned in the long-term, to be included in future effectoromics approaches and identify, for example, *Avr-Ph2*.

- Target enrichment and sequencing approaches could be developed for many other genes, including pathogen genes to simultaneously monitor effector diversity and the diversity of targets for chemical compounds.

References

Agrios GN (1997) Plant Pathology 4th Edition, London: Elsevier

Afzal AJ, Wood AJ, Lightfoot DA (2008) Plant receptor-like serine threonine kinases: roles in signaling and plant defense. Molecular Plant Microbe Interaction 21, 5, pp. 507 – 517.

Albrecht M., and Takken FL (2006) Update on the domain architectures of NLRs and R proteins. Biochemical and Biophysical Research Communications 339, pp. 459 – 462.

Allen RL, Meitz JC, Baumber RE, Hall SA, Lee SC, Rose LE and Beynon JL (2008) Natural variation reveals key amino acids in a downy meldew effector that alters recognition specificity by *Arabidopsis* resistance gene. Molecular Plant Pathology 9, pp. 511 – 523.

Andolfo G, Jupe F, Witek K, Etherington GJ, Ercolano MR and Jones JDG (2014) Defining the full tomato NB-LRR resistance gene repertoire using genomic and cDNA RenSeq. BMC Plant Biology Vol 14, 120. doi: 10.1186/1471-2229-14-120

Andolfo G, Sanseverino W, Rombauts S, Van de Peer Y, Bradeen JM, Carputo D, Frusciante L and Ercolano MR (2013) Overview of tomato (*Solanum lycopersicum*) candidate pathogen recognition genes reveals important *Solanum R* locus dynamics. New Phytologist 197, pp. 223 – 237.

Andrews S. (2010) FastQC: a quality control tool for high throughput sequence data. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc

Andrivon D, Avendano-Corcoles J, Cameron AM, Carnegie SF, Cooke LR, Corbiere R, Detourné D, Dowley LJ, Forisekova K and Griffin D (2011). Stability and variability of virulence of *Phytophthora infestans* assessed in a ring test across European laboratories. Plant Pathology, Vol 60, Issue 3, pp. 556 – 565.

Armstrong MR, Whisson SC, Pritchard L, Bos JIB, Venter E, Avrova AO, Rehmany AP, Böhme U, Brooks K, Cherevach I, Hamlin N, White B, Fraser A, Lord A, Quail MA, Churcher C, Hall N, Berriman M, Huang S, Kamoun S, Beynon JL, and Birch PRJ (2005) An ancestral oomycete locus contains late blight avirulence gene Avr3a, encoding a protein that is recognized in the host cytoplasm. PNAS 102, Issue 21, pp. 7766 – 7771.

Baker B, Zambryski P, Staskawicz B, Dinesh-Kumar SP (1997) Signaling in Plant-Microbe Interactions. Science, 276, pp. 726 – 733.

Ballvora A, Ercolano MR, Weiû J, Meksem K, Bormann CA, Oberhagemann P, Salamini F and Gebhardt C (2002) The R1 gene for potato resistance to late blight (Phytophthora infestans) belongs to the leucine zipper/NBS/LRR class of plant resistance genes. The Plant Journal 30(3), 361 – 371

Berendsen RL, Pieterse CM and Bakker PA (2012) The rhizosphere microbiome and plant health. Trends in Plant Science, Vol 17, Issue 8, pp. 478 – 486. doi: 10.1016/j.tplants.2012.04.00

Bergelson J, Kreitman M, Stahl EA and Tian D (2001) Evolutionary dynamics of plant *R*-genes. Science 292, pp. 2281 – 2285.

Birch PRJ, Bryan G, Fenton B, Gilroy EM, Hein I, Jones JT, Prashar A, Taylor MA, Torrance L and Toth IK (2012) Crops that feed the world 8: Potato: are the trends of increased global production sustainable? Food Security, Vol. 4, No. 4, 12.2012, p. 477-508. DOI 10.1007/s12571-012-0220-1

Birch PRJ, Boevink PC, Gilroy EM, Hein I, Pritchard L and Whisson SC (2008) Oomycete RxLR effectors: delivery, functional redundancy and durable disease resistance. Current Opinion in Plant Biology 11: 373 – 379.

Birch PRJ and Whisson SC (2001) *Phytophthora infestans* enters the genomics era. Molecular Plant Pathology 2, Issue 5, pp. 257 – 263.

Bonde R and Murphy EF (1952) Resistance of certain tomato varieties and crosses to late blight. Maine Agricultural Experiment Station 497, pp. 5 – 15.

Bos JIB, Armstrong MR, Gilroy EM, Boevink PC, Hein I, Taylor RM, Zhendong T, Engelhardt S, Vetukuri RR, Harrower B, Dixelius C, Bryan G, Sadanandom A, Whisson SC, Kamoun S, and Birch PRJ (2010) Phytophthora infestans effector AVR3a is essential for virulence and manipulates plant immunity by stabilizing host E3 ligase CMPG1. PNAS Vol 107, Number 21, pp. 9909 – 9914. doi: 10.1073/pnas.0914408107

Bos JIB, Kanneganti TD, Young C, Cakir C, Huitema E, Win J, Armstrong MR, Birch PRJ, and Kamoun S 2006. The C-terminal half of *Phytophthora infestans* RXLR effector *AVR3a* is sufficient to trigger *R3a*-mediated hypersensitivity and suppress INF1-induced cell death in *Nicotiana benthamiana*. The Plant Journal 48, pp. 165 – 76.

Bozkurt TO, Schornack S, Banfield MJ and Kamoun S (2012) Oomycetes, effectors, and all that jazz. Current Opinion in Plant Biology 15, pp. 1 – 10.

Bradshaw JE, Bryan GJ, Lees AK, McLean K and Solomon-Blackburn RM (2006a) Mapping the *R10* and *R11* genes for resistance to late blight (*Phytophthora infestans*) present in the potato (*Solanum tuberosum*) *R*-gene differentials of black. Theorical and Applied Genetics, Vol 112, Issue 4, pp. 744 – 751. doi: 10.1007/s00122-005-0179-9

Bradshaw JE, Bryan GJ and Ramsay G (2006b) Genetic Resources (Including Wild and Cultivated Solanum Species) and Progress in their Utilisation in Potato Breeding. Potato Research Vol 49, Issue 1, pp. 49 – 65. doi: 10.1007/s11540-006-9002-5

Bradshaw JE and Ramsay G (2005) Utilisation of the Commonwealth Potato Collection in potato breeding. Euphytica 146, pp.9 – 19. doi: 10.1007/s10681-005-3881-4

Bulgarelli D, Schlaeppi K, Spaepen S, Ver Loren van Themaat E and Schulze-Lefert P (2013) Structure and Functions of the Bacterial Microbiota of Plants. Annual Review of Plant Biology, 64, pp. 807 – 838. doi: 10.1146/annurev-arplant-050312-120106

Cárdenas M, Grajales A, Sierra R, Rojas A, González-Almario A, Vargas A, Marín M, Fermín G, Lagos LE, Grünwald NJ, Bernal A, Salazar C and Restrepo S (2011) Genetic diversity of Phytophthora infestans in the Northern Andean region. BMC Genetics, Volume 12, Issue 23. doi: 10.1186/1471-2156-12-23

Champouret N PhD thesis (2010) Functional genomics of *Phytophthora infestans* effectors and *Solanum* resistance genes.

Champouret N, Bouwmeester K, Rietman H, van der Lee T, Maliepaard C, Heupink A, van de Vondervoort PJ, Jacobsen E, Visser RG, van der Vossen EA, Govers F and Vleeshouwers VG

(2009) *Phytophthora infestans* isolates lacking class I *ipiO* variants are virulent on Rpi-blb1 potato. MPMI 22, pp. 1535 – 1545.

Chapman S and Stevens LJ Boevink PC, Engelhardt S, Alexander CJ, Harrower B, Champouret N, McGeachy K, Van Weymers PSM, Chen X, Birch PRJ and Hein I (2014) Detection of the Virulent Form of AVR3a from *Phytophthora infestans* following Artificial Evolution of Potato Resistance Gene *R3a*. PLoS ONE Vol. 9, Issue 10: e110158. doi:10.1371/journal.pone.0110158

Chisholm ST, Coaker G, Day B, and Staskawicz BJ (2006) Host-microbe interactions: shaping the evolution of the plant immune response. Cell 124, Issue 4, pp. 803 – 814.

Chunwongse J, Chunwongse C, Black L and Hanson P (2002) Molecular mapping of the *Ph-3* gene for late blight resistance in tomato. Journal of Horticultural Science & Biotechnology 77, Issue 3, pp. 281 – 286.

Cohen Y, and Reuveni M (1983). Occurence of metalaxyl-resistance isolates of *Phytophthora infestans* in potato field in Israel. Phytopathology 73, pp. 925 – 927.

Cooke DEL, Cano LM, Raffaele S, Bain RA, Cooke LR, Etherington GJ, Deahl KL, Farrer RA, Gilroy EM, Goss EM, Grünwald NJ, Hein I, MacLean D, McNicol JW, Randall E, Oliva RF, Pel MA, Shaw DS, Squires JN, Taylor MC, Vleeshouwers VGAA, Birch PRJ, Lees AK, Kamoun S (2012) Genome analyses of an aggressive and invasive lineage of the Irish potato famine pathogen. PLOS pathogen 8:e1002940.

Cooke LR (1981). Resistance to metalaxyl in *Phytophthora infestans* in northern Ireland. Proceedings of the Brighton Crop Protection Conference, Pests and Diseases, pp. 641 – 650.

Cronn R, Knaus BJ, Liston A, Maughan PJ, Parks M, Syring JV and Udall J (2012) Targeted enrichment strategies for next-generation plant biology. American Journal of Botany, Vol 99, Number 2, pp. 291 – 311. doi: 10.3732/ajb.1100356

Cruickshank G, Stewart HE and Wastie RL (1982) An illustrated assessment key for foliage blight of potatoes. Potato Research 25, pp. 213 – 214.

Damasceno CMB, Bishop JG, Ripoll DR, Win J, Kamoun S, and Rose JKC (2008) Structure of the glucanase inhibitor protein (GIP) family from *Phytophthora* species suggests coevolution with plant endo-beta-1,3-glucanases. Molecular Plant Microbe Interaction 21, pp. 820 – 830.

Dangl JL and Jones JDG (2001) Plant pathogens and integrated defence responses to infection. Nature 411, pp. 826 – 833.

Dangl JL, Dietrich RA, and Richberg MH (1996) Death don‘t have no mercy: cell death programs in plant-microbe interactions. Plant Cell 8, pp. 1793 – 1807.

Davidse LC, Looijen D, Turkensteen LJ, and Vanderwal D (1981) Occurence of metalaxyl-resistance strains of *Phytophthora infestans* in Dutch potato fields. Netherlands Journal of Plant Pathology 87, pp. 65 – 68.

Deahl KL, Cooke LR, Black LL, Wang TC, Perez FM, Moravec BC, Quinn M, and Jones RW (2002) Population changes in *Phytophthora infestans* in Taiwan associated with the appearance of resistance to metalaxyl. Pest Management Science 58, pp. 951 – 958.

Deahl KL, Demuth SP, Pelter G, and Ormrod DJ (1993) 1st report of resistance of *Phytophthora infestans* to metalaxyl in eastern Washington and southwestern British-Columbia. Plant Disease 77, pp. 429 – 429.

Devos KM, Brown JKM, and Bennetzen JL (2002) Genome size reduction through illegitimate recombination counteracts genome expansion in *Arabidopsis*. Genome Research 12, pp. 1075 – 1079.

Diévart A and Clark SE (2004) LRR-containing receptors regulating plant development and defense. Development 131, pp. 251 – 261.

Ding J, Zhang Z, Chen JQ and Tian D (2007) Unique pattern of *R*-gene variation within populations in *Arabidopsis*. Molecular Genetics and Genomics 277, pp. 619 – 629.

Dodds PN, Lawrence GJ, Catanzariti AM, Teh T, Wang CI, Ayliffe MA, Kobe B, and Ellis JG (2006) Direct protein interaction underlies gene-for-gene specificity and coevolution of the flax resistance genes and flax rust avirulence genes. Proceedings of the National Academy of Sciences of the United States of America 103, pp. 8888 – 8893.

El Kharbotly A, Palomino-Sánchez C, Salamini F, Jacobsen E and Gebhardt C (1996) R6 and R7 alleles of potato conferring race-specific resistance to Phytophthora infestans (Mont.) de Bary identified genetic loci clustering with the R3 locus on chromosome XI. Theoretical and Applied Genetics, Vol 92, Issue 7, pp. 880 – 884. doi: 10.1007/BF00221901

El Kharbotly A, Leonards-Schippers C, Huigen DJ, Jacobsen E, Pereira A, Stiekema WJ, Salamini F, and Gebhardt C (1994) Segregation analysis and RFLP mapping of the *R1* and *R3* alleles conferring race-specific resistance to *Phytophthora infestans* in progeny of dihaploid potato parents. Molecular Genetics and Genomics 242, 6, pp. 749 – 754.

Ellis J G, Lawrence G J, and Dodds PN (2007a) Flax rust resistance specificity is based on direct resistance-avirulence protein interactions. Annual review of Phytopathology 45, pp.289 – 306.

Ellis J G, Lawrence G J, and Dodds PN (2007b) Further analysis of gene-for-gene disease resistance specificity in flax. Molecular Plant Pathology 8, pp. 103 – 109.

Erwin DC and Ribeiro OK (1996) *Phytophthora* diseases worldwide.

Flor HH (1971) Current status of the gene-for-gene concept. Annual Review in Phytopathology 9, pp. 275 – 296.

Foolad MR, Merk HL and Ashrafi H (2008) Genetics, genomics and breeding of late blight and early blight resistance in tomato. Critical Reviews in Plant Sciences 27: 2, pp. 75 – 107.

Foster SJ, Park T-H, Pel M, Brigneti G, Sliwka J, Jagger L, van der Vossen E and Jones JDG (2009) Rpi-vnt1.1, a Tm-22 Homolog from Solanum venturii, confers resistance to potato late blight. MPMI Vol 22, Issue 5, pp. 598 – 600. doi: 10.1094/MPMI-22-5-0589.

Friedman AR, and Baker BJ (2007) The evolution of resistance genes in multi-protein plant resistance systems. Current Opinion in Genetics and Development 17, pp. 493 – 499.

Fry WE, McGrath MT, Seaman A, Zitter TA, McLeod A, Danies G, Small IM, Myers K, Everts K, Gevens AJ, Gugino BK, Johnson SB, Judelson SB, Ristaino J, Roberts P, Secor G, Seebold K Jr., Snover-Clift K, Wyenandt A, Grünwald NJ and Smart CD (2009) The 2009 late blight pandemic in the Eastern United States - causes and results. Plant Disease 97, Issue 3, pp. 296 – 306.

Fry WE (2008) *Phytophthora infestans*: the plant (and *R* gene) destroyer. Molecular Plant Pathology 9, pp. 385 – 402.

Fry WE and Goodwin SB (1997a) Resurgence of the Irish potato famine fungus. BioScience 47, pp. 363 – 371.

Fry WE and Goodwin SB (1997b) Re-emergence of potato and tomato late blight in the United States. Plant Disease 81, pp. 1349 – 1357.

Gallegly ME and Marvel ME (1955) Inheritance of resistance to tomato race-0 of *Phytophthora infestans*. Phytopathology 45, pp. 103 – 109.

Gilroy EM, Breen S, Whisson SC, Squires J, Hein I, Kaczmarek M, Turnbull D, Boevink PC, Lokossou A, Cano LM, Morales J, Avrova AO, Pritchard L, Randall E, Lees A, Govers F, van West P, Kamoun S, Vleeshouwers VGAA, Cooke DEL and Birch PRJ (2011) Presence/absence, differential expression and sequence polymorphisms between PiAVR2 and PiAVR2-like in Phytophthora infestans determine virulence on R2 plants. New Phytologist 191(3): 763 – 776. DOI: 10.1111/j.1469-8137.2011.03736.x

Gish LA and Clark SE (2011) The RLK/Pelle family of kinases. The Plant Journal 66, pp. 117 – 127.

Golas TM, van de Geest H, Gros J, Sikkema A, D'Agostino N, Nap JP, Mariani C, Allefs JJHM and Rieu I (2013) Comparative next-generation mapping of the *Phytophthora infestans* resistance gene *Rpi-dlc2* in a European accession of *Solanum dulcamara*. Theoretical and Applied Genetics 126, pp. 59 – 68. doi: 10.1007/s00122-012-1959-7

Golas TM, Sikkema A, Gros J, Feron RMC, van den Berg RG, van der Weerden GM, Mariani C and Allefs JJHM (2010) Identification of a resistance gene *Rpi-dlc1* to *Phytophthora infestans* in European accessions of *Solanum dulcamara*. Theoretical and Applied Genetics 120, pp. 797 – 808. doi: 10.1007/s00122-009-1202-3

Gomez-Alpizar L, Carbone I, and Ristaino JB (2007) An Andean origin of *Phytophthora infestans* inferred from mitochondrial and nuclear gene genealogies. Proceedings of the National Academy of Sciences of the United States of America 104, pp. 3306 – 3311.

Gonzalez-Lamothe R, Mitchell G, Gattuso M, Diarra MS, Malouin F, and Bouarab K (2009) Plant antimicrobial agents and their effects on plant and human pathogens. International Journal of Molecular Sciences 10, pp. 3400 – 3419.

Goodwin SB and Drenth A (1997) Origin of the A2 mating type of *Phytophthora infestans* outside Mexico. Phytopathology 87, Issue 10, pp. 992 – 999.

Goodwin SB, Sujkowski LS and Fry WE (1995) Rapid evolution of pathogenicity within clonal lineages of the potato late blight disease fungus. Phytopathology 85, pp. 669 – 676.

Goss EM, Tabima JF, Cooke DEL, Restrepo S, Fry WE, Forbes GA, Fieland VJ, Cardenas M and Grünwald NJ (2014) The Irish potato famine pathogen Phytophthora infestans originated in central Mexico rather than the Andes. PNAS Vol 111, Number 24, pp. 8791 – 8796. doi: 10.1073/pnas.1401884111

Grünwald NJ and Flier WG (2005) the biology of *Phytophthora infestans* at its center of origin. Annual Review of Phytopathology, pp. 171 – 190.

Haas BJ, Kamoun S, Zody MC, Jiang RHY, Handsaker R, Cano LM, Grabherr M, Kodira CD, Raffaele S, Torto-Alalibo T, Bozkurt TO, Ah-Fong AMV, Alvarado L, Anderson VL, Armstrong MR, Avrova A, Baxter L, Beynon J, Boevink PC, Bollmann SR, Bos JIB, Bulone V, Cai G, Cakir C, Carrington JC, Chawner M, Conti L, Costanzo S, Ewan, R, Fahlgren N, Fischbach MA, Fugelstad J, Gilroy EM, Gnerre S, Green PJ, Grenville-Briggs LJ, Griffith J, Grunwald NJ, Horn K, Horner NR, Hu CH, Huitema H, Jeong DH, Jones AME, Jones DGJ, Jones RW, Karlsson EK, Kunjeti SG, Lamour K, Liu Z, Ma L, MacLean D, Chibucos MC, McDonald H, McWalters J, Meijer HJG, Morgan W, Morris PF, Munro CA, O'Neill K, Ospina-Giraldo M, Pinzon A, Pritchard L, Ramsahoye B, Ren Q, Restrepo S, Roy S, Sadanandom a, Sadivor A, Schornack S, Schwartz DC, Schumann UD, Schwessinger B, Seyer L, Sharpe T, Silvar C, Song J, Studholme DJ, Sykes S, Thines M, Van de Vondervoort PJI, Phuntumart V, Wawra S, Weide R, Win J, Young C, Zhou S, Fry W, Meyers BC, Van West P, Ristaino J, Govers F, Birch PRJ, Whisson SC, Judelson HS and Nusbaum C (2009) Genome sequence and analysis of the Irish potato famine pathogen Phytophthora infestans. Nature 461, pp. 393 – 398.

Hanneman RE (1999) The reproductive biology of the potato and its implication for breeding. Potato Research 42S, pp. 283 – 312. doi: 10.1007/BF02357859

Haverkort AJ, Boonekamp PM, Hutten R, Jacobsen E, Lotz LAP, Kessel GJT, Vossen JH and Visser RGF (2016) Durable Late Blight Resistance in Potato Through Dynamic Varieties Obtained by Cisgenesis: Scientific and Societal Advances in the DuRPh Project. Potato Research Vol 59, Issue 1, pp 35 – 66. doi: 10.1007/s11540-015-9312-6

Haverkort A, Struik P, Visser R and Jacobsen E (2009) Applied biotechnology to combat late blight in potato caused by Phytophthora infestans. Potato Research 52: 249 – 264. doi:10.1007/s11540-009-9136-3

Haverkort AJ, Boonekamp PM, Hutten R, Jacobsen E, Lotz LAP, Kessel GJY, Visser RGF and van der Vossen EAG (2008) Societal Costs of Late Blight in Potato and Prospects of Durable Resistance Through Cisgenic Modification. Potato Research 51, pp. 47 – 57. doi: 10.1007/s11540-008-9089-y

Hayden AC (2014) The $1,000 genome. Nature Vol 507, Issue 7492, pp: 294 – 295. doi:10.1038/507294a

Hawkes J (1990) The potato: evolution, biodiversity, and genetic resources. Belhaven Press, London.

Hein I, Birch PRJ, Danan S, Lefebvre V, Achieng Odeny D, Gebhardt C, Trognitz F, and Bryan G (2009a) Progress in mapping and cloning qualitative and quantitative resistance against *Phytophthora infestans* in potato and its wild relatives. Potato Research 52, pp. 215 – 227.

Hein I, Gilroy EM, Armstrong MR and Birch PRJ (2009b) The zig-zag-zig in oomycete-plant interactions. Molecular Plant Pathology 10 (4): 547 – 562. doi: 10.1111/j.1364-3703.2009.00547.x.

Hein I, McLean K, Chalhoub B, Bryan GJ (2007) Generation and screening of a BAC library from a diploid potato clone to unravel durable late blight resistance on linkage group IV. Internal Journal of Plant Genomics, Vol 2007, ID 51421. doi: 10.1155/2007/51421

Helgeson JP, Pohlman JD, Austin S, Haberlach GT, Wielgus SM, Ronis D, Zambolim L, Tooley P, McGrath JM, James RV and Stevenson WR (1998) Somatic hybrids between *Solanum bulbocastanum* and potato: a new source of resistance to late blight. Theoretical and Applied Genetics Vol 96, Issue 6, pp: 738 – 742. doi: 10.1007/s001220050796

Hermsen JGT, Ramanna MS. 1973. Double-bridge hybrids of *Solanum bulbocastanum* and cultivars of *Solanum tuberosum*. Euphytica 22, pp. 457 – 66. doi: 10.1007/BF00036641

Huang S, van der Vossen EA, Kuang H, Vleeshouwers VG, Zhang N, Borm TJ, van Eck HJ, Baker B, Jacobsen E, and Visser RG (2005) Comparative genomics enabled the isolation of the R3a late blight resistance gene in potato. Plant Journal 42, pp. 251 – 261.

Huang S, Vleeshouwers VG, Werij JS, Hutten RC, van Eck HJ, Visser RG, and Jacobsen E (2004). The *R3* resistance to *Phytophthora infestans* in potato is conferred by two closely linked R genes with distinct specificities. Molecular Plant Microbe Interaction 17, pp. 428 – 435.

Hückelhoven R (2007) CellWall–Associated mechanisms of disease resistance and susceptibility. Annual review of Phytopathology, 45, pp. 101 – 127.

Irzhansky I and Cohen Y (2006) Inheritance of resistance against *Phytophthora infestans* in *Lycopersicon pimpenellifolium* L3707. Euphytica 149, pp. 309 – 316.

Jacobs MMJ, Vosman B, Vleeshouwers VGAA, Visser RGF, Henken B and van den Berg RG (2010) A novel approach to locate *Phytophthora infestans* resistance genes on the potato genetic map. Theoretical and Applied Genetic, Vol 120, Issue 4, pp. 785 – 796. doi: 10.1007/s00122-009-1199-7

Jacobs MMJ, Van den Berg R, Vleeshouwers V, Visser M, Mank R, Sengers M, Hoekstra R and Vosman B (2008) AFLP analysis reveals a lack of phylogenetic structure within *Solanum* section *Petota*. BMC Evolutionary Biology 8, pp. 145 – 157.

Jacobs MMJ PhD thesis (2008) Searching for species, relationships and resistance in *Solanum* section *Petota*.

Jacobsen E and Hutten R (2006) Stacking resistance genes in potato by cisgenesis instead of introgression breeding. Potato developments in a changing Europe, Edited by NU Haase and AJ Haverkort, Wageningen Academic Publishers. doi: 10.3920/978-90-8686-582-6

Jiang RHY, Weide R, van de Vondervoort PJI, Govers F (2006) Amplification generates modular diversity at an avirulence locus in the pathogen *Phytophthora*. Genome Research, Vol 16(7), pp. 827 – 840. doi:10.1101/gr.5193806.

Jo KR, Visser RGF, Jacobsen E and Vossen JH (2015) Characterisation of the late blight resistance in potato differential *MaR9* reveals a qualitative resistance gene, *R9a*, residing in a cluster of *Tm-2²* homologs on chromosome IX. Theoretical and Applied Genetics, Vol 128, Issue 5, pp. 931 – 941. doi: 10.1007/s00122-015-2480-6

Jo KR, Kim CJ, Kim SJ, Kim TY, Bergervoet M, Jongsma MA, Visser RGF, Jacobsen E and Vossen JH (2014) Development of late blight resistant potatoes by cisgene stacking. BMC Biotechnology, Vol 14, Issue 50. doi: 10.1186/1472-6750-14-50

Jo KR, Arens M, Kim TY, Jongsma MA, Visser RGF, Jacobsen E and Vossen JH (2011) Mapping of the *S. demissum* late blight resistance gene *R8* to a new locus on chromosome IX. Theoretical and Applied Genetics 123, pp. 1331 – 1340.

Jones JDG, Witek K, Verweij W, Jupe F, Cooke D, Dorling S, Tomlinson L, Smoker M, Perkins S and Foster S (2014) Elevating crop disease resistance with cloned genes. Philosophical Transactions of the Royal Society B, 369 (19639): 20130087. doi: 10.1098/rstb.2013.0087

Jones JDG and Dangl JL (2006) The plant immune system. Nature 444, 323–329. doi: 10.1038/nature05286

Jones JDG, Foster SJ, Chu Z, Park TH, van der Vossen EAG, Pel MA , Gerardus R and Visser F (2009) Late blight resistance genes and methods. Patent application WO2009013468.

Jones DA, Jones JDG (1997) The role of Leucine-Rich Repeat proteins in plant defences. Advances in Botanical Research. Academic Press, pp. 89 – 167.

Jupe F, Pritchard L, Etherington GJ, MacKenzie K, Cock PJA, Wright F, Sharma SK, Bolser D, Bryan GJ, Jones JDG and Hein I (2012) Identification and localisation of the NB-LRR gene family within the potato genome. BMC Genetics 13: 75Jones JDG and Dangl JL (2006) The plant immune system. Nature 444, pp. 323 – 329 PMID:17108957

Jupe F, Witek K, Verweij W, Sliwka J, Pritchard L, Etherington GJ, Maclean D, Cock PJ, Leggett RM, Bryan GJ, Cardle L, Hein I and Jones JDG (2013) Resistance gene enrichment sequencing (RenSeq) enables reannotation of the NB-LRR gene family from sequenced plant genomes and rapid mapping of resistance loci in segregating populations. The Plant Journal 76, pp. 530 – 544 doi: 10.1111/tpj.12307

Jupe F, Chen X,Verweij W, Witek K, Jones JD and Hein I (2014) Genomic DNA library preparation for resistance gene enrichment and sequencing (RenSeq) in plants. Methods in Molecular Biology 1127, pp. 291 – 303. doi: 10.1007/978-1-62703-986-4_22

Kamoun S (2006) A catalogue of the effector secretome of plant pathogenic oomycetes. Annual Review in Phytopathology 44, pp. 41 – 60.

Kim MJ and Mutschler MA (2005) Transfer to processing tomato and characterisation of late blight resistance derived from *Solanum pimpinellifolium* L. L3708. Journal of the American Society for Horticultural Science 131, pp. 637 – 645.

Kinkel LL, Bakker MG and Schlatter DC (2011) A Coevolutionary Framework for Managing Disease-Suppressive Soils. Annual Review of Phytopathology, Vol 49, pp. 47 – 67. doi: 10.1146/annurev-phyto-072910-095232

Kuang HH, Woo SS, Meyers BC, Nevo E, and Michelmore RW (2004) Multiple genetic processes result in heterogeneous rates of evolution within the major cluster disease resistance genes in lettuce. Plant-Cell 16, pp. 2870 – 2894.

Kuhl JC, Hanneman RE Jr and Havey MJ (2001) Characterization and mapping of *Rpi1*, a late-blight resistance locus from diploid (1EBN) Mexican *Solanum pinnatisectum*. Molecular Genetics and Genomics, Vol 265, Issue 6, pp. 977 – 985.

Lanfermeijer FC, Dijkhuis J, Sturre MJ, de Haan P and Hille J (2003) Cloning and characterization of the durable tomato mosaic virus resistance gene *Tm-2(2)* from *Lycopersicon esculentum*. Plant Molecular Biology, Vol 52, Issue 5, pp. 1037 – 1049.

Langmead B and Salzberg SL (2012) Fast gapped-read alignment with Bowtie2. Nature Methods 9, pp. 357-359. doi:10.1038/nmeth.1923

Leister D (2004). Tandem and segmental gene duplication and recombination in the evolution of plant disease resistance genes. Trends in Genetics, Vol 20, pp. 116 – 122.

Lenman M, Ali A, Mühlenbock P, Carlson-Nilsson U, Liljeroth E, Champouret N, Vleeshouwers VGAA and Andreasson E (2016) Effector-driven marker development and cloning of

resistance genes against Phytophthora infestans in potato breeding clone SW93-1015. Theoretical and Applied Genetics 129:105–115. DOI 10.1007/s00122-015-2613-y

Li J, Xie C,Tian Z, Lindqvist-Kreuze H, Bonierbale M and Liu J (2015) SSR and e-PCR Provide a Bridge Between Genetic Map and Genome Sequence of Potato for Marker Development in Target QTL Region. American Journal of Potato Research, Vol 92, pp. 312 – 317. doi: 10.1007/s12230-015-9432-1

Li G, Huang S, Guo X, Li Y, Yang Y, Guo Z, Kuang H, Rietman H, Bergervoet M, Vleeshouwers VGGA, van der Vossen EAG, Qu D, Visser RGF, Jacobsen E, and Vossen JH (2011) Cloning and characterization of R3b; members of the R3 superfamily of late blight resistance genes show sequence and functional divergence. MPMI 24, Number 10, pp. 1132–1142. doi:10.1094 / MPMI -11-10-0276

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, and 1000 Genome Project Data Processing Subgroup (2009) The Sequence alignment/map (SAM) format and SAMtools. Bioinformatics 25, Issue 16, pp. 2078 – 2079. doi: 10.1093/bioinformatics/btp352

Li X, van Eck HJ, Rouppe van der Voort JNAM, Huigen DJ, Stam P and Jacobsen E (1998) Autotetraploids and genetic mapping using common AFLP markers: the *R2* allele conferring resistance to *Phytophthora infestans* mapped on potato chromosome 4. Theoretical and Applied Genetics, Vol 96, Issue 8, pp 1121 – 1128. doi: 10.1007/s001220050847

Lokossou AA, Rietman H, Wang M, Krenek P, van der Schoot H, Henken B, Hoekstra R, Vleeshouwers VGAA, van der Vossen EAG, Visser RGF, Jacobsen E, and Vosman B (2010) Diversity, distribution, and evolution of Solanum bulbocastanum late blight resistance genes. MPMI Vol 23, Number 9, pp. 1206 – 1216. doi:10.1094/MPMI -23-9-1206.

Lokossou AA, Park TH, van Arkel G, Arens M, Ruyter-Spira C, Morales J, Whisson SC, Birch PRJ, Visser RGF, Jacobsen E and Van der Vossen EAG (2009) Exploiting knowledge of R/Avr genes to rapidely clone a new LZ-NBS-LRR family of late blight resistance genes from potato linkage group IV. MPMI 22, Issue 6, pp. 630 – 641.

Luderer R, Takken FLW, de Wit PJGM and Joosten MHAJ (2002) *Cladosporium fulvum* overcomes Cf-2-mediated resistance by producing truncated AVR2 elicitor proteins. Molecular Microbiology, Vol 45 , Issue 3, pp. 875 – 884. doi: 10.1046/j.1365-2958.2002.03060.x

Lupas A (1996) Coiled coils: new structures and new functions. Trends in Biochemical Sciences 21, Issue 10, pp. 375 – 382.

Malcolmson J F (1969) Races of Phytophthora infestans occurring in Great Britain. Transactions of the British Mycological Society. 53, Issue 3, pp. 417 – 423.

Martin M (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.Journal, 17(1):10-12. http://dx.doi.org/10.14806/ej.17.1.200

Matsushima N and Miyashita H (2012) Leucine-Rich Repeat (LRR) domains containing intervening motifs in plants. Biomolecules 2, pp. 288 – 311.

McDonald BA., and Linde C (2002) Pathogen population genetics, evolutionary potential, and durable resistance. Annual Review of Phytopathology pp. 349 – 379.

McHale L, Tan X, Koehl P, and Michelmore RW (2006) Plant NBS-LRR proteins: adaptable guards. Genome Biology 7: 212.

Medzhitov R and Janeway CA Jr. (1997) Innate Immunity: the virtues of a nonclonal system of recognition. Cell 91, 3, pp. 295 – 298.

Mendes R, Kruijt M, de Bruijn I, Dekkers E, van der Voort M, Schneider JH, Piceno YM, DeSantis TZ, Andersen GL, Bakker PA and Raaijmakers JM (2011) Deciphering the rhizosphere microbiome for disease-suppressive bacteria. Science, Vol 332, Issue 6033, pp. 1097 – 1100. doi: 10.1126/science.1203980

Meyers BC, Kozik A, Griego A, Kuang H and Michelmore RW (2003) Genome-wide analysis of NBS-LRR–encoding genes in *Arabidopsis*. The Plant Cell 15, pp. 809 – 834.

Meyers BC, Dickerman AW, Michelmore RW, Sivaramakrishnan S, Sobral BW, and Young ND (1999) Plant disease resistance genes encode members of an ancient and diverse protein family within the nucleotide-binding superfamily. Plant Journal 20, pp. 317 – 332. PMID:10571892

Meyers BC, Shen KA, Rohani P, Gaut BS and Michelmore RW (1998) Receptor-like genes in the major resistance locus of lettuce are subject to divergent selection. The Plant Cell 11, pp. 1833 – 1846.

Michelmore RW, Paran I and Kesseli RV (1991) Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. PNAS, Vol 88, pp. 9828 – 9832.

Miller JS, Hamm PB, and Johnson DA (1997) Characterization of the *Phytophthora infestans* population in the Columbia Basin of Oregon and Washington from 1992 to 1995. Phytopathology 87, pp. 656 – 660.

Moreau P, Thoquet P, Olivier J, Laterrot H and Grimsley N (1998) Genetic mapping of *Ph-2*, a single locus controlling partial resistance to *Phytophthora infestans* in tomato. MPMI 11, Number 4, pp. 259 – 269.

Mukerji KG (2004) Fruit and Vegetable Diseases. Boston: Kluwer Academic Publishers.

Naess SK, Bradeen JM, Wielgus SM, Haberlach GT, McGrath JM, Helgeson JP (2000) Resistance to late blight in *Solanum bulbocastanum* is mapped to chromosome 8. Theoretical and Applied Genetics 101, pp. 697 – 704. doi: 10.1007/s001220051533

Nieks (2011).

Nombela G, Williamson VM and Muniz M (2003) The root-knot nematode resistance gene *Mi-1.2* of tomato is responsible for resistance against the whitefly Bemisia tabaci. MPMI Vol 16, Number 7, pp. 645 – 649. doi: 10.1094/MPMI.2003.16.7.645

Nowicki M, Foolad MR, Nowakowska M and Kozik EU (2012) Potato and tomato late blight caused by *Phytophthora infestans*: an overview of pathology and resistance breeding. Plant disease 96, pp. 4 – 17.

Oh SK., Young C, Lee M, Oliva R, Bozkurt TO, Cano LM, Win J, Bos JIB, Liu H-Y, van Damme M, Morgan W, Choi D, Van der Vossen EAG, Vleeshouwers VGA, and Kamoun S (2009). *In Planta* expression screens of *Phytophthora infestans* RXLR effectors reveal diverse phenotypes,

including activation of the *Solanum bulbocastanum* disease resistance protein *Rpi-blb2*. Plant Cell 21, Issue 9, pp. 2928 – 2947.

Park TH, Vleeshouwers VGAA, Jacobsen E, van der Vossen E, Visser RGF (2009) Molecular breeding for resistance to *Phytophthora infestans* (Mont.) de Bary in potato (Solanum tuberosum L.): a perspective of cisgenesis. Plant Breed 128, pp. 109–117. doi: 10.1111/j.1439-0523.2008.01619.x

Park TH, Gros J, Sikkema A, Vleeshouwers VGAA, Muskens M, Allefs S, Jacobsen E, Visser RGF and Van der Vossen EAG (2005) The late blight resistance locus *Rpi-blb3* from *Solanum bulbocastanum* belongs to a major late blight *R* gene cluster on chromosome 4 of potato. Molecular Plant-Microbe Interactions 18, Number 7, pp. 722 – 729.

Pel MA, Foster SJ, Park TH, Rietman H, van Arkel G, Jones JDG, Van Eck HJ, Jacobsen E, Visser RGF and Van der Vossen EGA (2009) Mapping and cloning of late blight resistance genes from Solanum venturii using an interspecific candidate gene approach. MPMI 22, Issue 5, pp. 601 – 615.

Pel MA (2010) Mapping, isolation and characterization of genes responsible for late blight resistance in potato. Ph.D. thesis, Wageningen University, Wageningen, The Netherlands.

Peirce LC (1971) Linkage tests with Ph conditioning resistance to race 0, Phytophthora infestans. Tomato Genetics Cooperative Report, 21: 30.

Potato Genome Sequencing Consortium (2011) Genome sequence analysis of the tuber crop potato. Nature 475, pp. 189 – 197.

Quarrie SA, Lazić-Jancić V, Kovacević D, Steed A and Pekic S (1999) Bulk segregant analysis with molecular markers and its use for improving drought resistance in maize. Journal of Experimental Botany, Vol 50, Number 337, pp. 1299 – 1306.

Quinlan AR and Hall IM (2010) BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics 26, Issue 6, pp. 841 – 842. doi: 10.1093/bioinformatics/btq033

Qureshi ST, Larivière L, Leveque G, Clermont S, Moore KJ, Gros P and Malo D (1999) Endotoxin-tolerant mice have mutations in toll-like receptor 4 (*Tlr4*). The Journal of Experimental Medicine 189, 4, pp. 615 – 625.

Raffaele S, Farrer RA, Cano LM, Studholme DJ, MacLean D, Thines M, Jiang RH, Zody MC, Kunjeti SG, Donofrio NM, Meyers BC, Nusbaum C, Kamoun S (2010) Genome evolution following host jumps in the Irish potato famine pathogen lineage. Science 330, pp. 1540 – 1543. doi: 10.1126/science.1193070

Rauscher GM, Smart CD, Simko I, Bonierbale M, Mayton H, Greenland A and Fry WE (2006) Characterisation and mapping of *Rpi-ber*, a novel potato late blight resistance gene from *Solanum berthaultii*. Theoretical and Applied Genetics 112, pp. 674 – 687.

Rice CM, Levis R, Strauss JH and Huang HV (1987). Production of infectious RNA transcripts from sindbis virus cDNA clones: mapping of lethal mutations, rescue of a temperature-sensitive marker, and in vitro mutagenesis to generate defined mutants. Journal of Virology 61, pp. 3809 – 3819.

Rick CM (1978) The tomato. *Science American*, 23, pp. 76 – 87.

Rodríguez F, Ghislain M, Clausen AM, Jansky SH and Spooner DM. 2010. Hybrid origins of cultivated potatoes. Theoretical and Applied Genetics 121, pp. 1187 – 1198.

Römer P, Hahn S., Jordan T, Strauss T, Bonas U and Lahaye T (2007) Plant pathogen recognition mediated by promoter activation of the pepper Bs3 resistance gene. Science 318, 5850, pp. 645 – 648.

Rooney HCE, van't Klooster JW, van der Hoorn RAL, Joosten MHAJ, Jones JDG and de Wit PJGM (2005) Cladosporium Avr2 Inhibits Tomato Rcr3 Protease Required for Cf-2-Dependent Disease Resistance. Science, Vol 308, Issue 5729, pp. 1783-1786. doi: 10.1126/science.1111404

Rossi M, Goggin FL, Milligan SB, Kaloshian I, Ullman DE and Williamson VM (1998) The nematode resistance gene *Mi* of tomato confers resistance against the potato aphid. PNAS Vol 95, Number 17, pp. 9750 – 9754.

Rossman AY and Palm ME (2006) Why are *phytophthora* and other *Oomycota* not true fungi? The American Phytopathological Society.

Sandbrink JM, Colon LT, Wolters PJCC and Stiekema WJ (2000) Two related genotypes of *Solanum Microdontum* carry different segregating alleles for field resistance to *Phytophthora infestans*. Molecular Breeding, pp. 215 – 225.

Schumann GL (1991) Plant Diseases: Their Biology and Social Impact.

Sharma SK, Bolser D, de Boer J, Sønderkær M, Amoros W, Carboni MF, D'Ambrosio JM, de la Cruz G, Di Genova A, Douches DS, Eguiluz M, Guo X, Guzman F, Hackett CA, Hamilton JP, Li G, Li Y, Lozano R, Maass A, Marshall D, Martinez D, McLean K, Mejía N, Milne L, Munive S, Nagy I, Ponce O, Ramirez M, Simon R, Thomson SJ, Torres Y, Waugh R, Zhang Z, Huang S, Visser RGF, Bachem CWB, Sagredo B, Feingold SE, Orjeda G, Veilleux RE, Bonierbale M, Jacobs JME, Milbourne D, Martin DMA and Bryan GJ (2013) Construction of reference chromosome-scale pseudomolecules for potato: integrating the potato genome with genetic and physical maps. G3 3, Issue 11, pp. :2031-2047. doi: 10.1534/g3.113.007153

Sliwka J, Jakuczun H, Chmielarz M, Hara-Skrzypiec A, Tomczyńska I, Kilian A and Zimnoch-Guzowska E (2012) A resistance gene against potato late blight originating from *Solanum × michoacanum* maps to potato chromosome VII. Theoretical and Applied Genetics, Vol 124, Issue 2, pp. 397 – 406. doi: 10.1007/s00122-011-1715-4

Sliwka J, Jakuczun H, Lebecka R, Marczewski W, Gebhardt C and Zimnoch-Guzowska E (2006) The novel, major locus *Rpi-phu1* for late blight resistance maps to potato chromosome IX and is not correlated with long vegetation period. Theoretical and Applied Genetics, Vol 113, Issue 4, pp. 685 – 695. doi : 10.1007/s00122-006-0336-9

Smilde WD, Brigneti G, Jagger L, Perkins S and Jones JDG (2005) *Solanum mochiquense* chromosome IX carries a novel late blight resistance gene *Rpi-moc1*. Theoretical and Applied Genetics, Vol 110, pp. 252 – 258. doi : 10.1007/s00122-004-1820-8

Song J, Win J, Tian M, Schornack S, Kaschani F, Ilyas M, van der Hoorn RAL and Kamoun S (2008) Apoplastic effectors secreted by two unrelated eukaryotic plant pathogens target the tomato defense protease Rcr3. PNAS, vol. 106, no. 5, pp. 1654–1659. doi: 10.1073/pnas.0809201106

Song J, Bradeen JM, Naess SK, Raasch JA, Wielgus SM, Haberlach GT, Liu J, Kuang H, Austin-Phillips S, Buell CR, Helgeson JP, and Jiang J (2003) Gene *RB* cloned from *Solanum*

bulbocastanum confers broad spectrum resistance to potato late blight. Proceedings of the National Academy of Sciences of the United States of America 100, pp. 9128 – 9133.

Spoel SH and Dong X (2012) How do plants achieve immunity? Defence without specialized immune cells. Nature Reviews 12, pp. 89 – 100.

Spooner DM, and Salas A (2006) Structure, biosystematics, and genetic resources. Handbook of potato production, improvement, and post-harvest management, pp. 1 – 39.

Spooner D, and Hijmans R (2001) Potato systematics and germplasm collecting, 1989–2000. American Journal of Potato Research 78, pp. 237 – 268.

Stahl EA, Dwyer G, Mauricio R, Kreitman M and Bergelson J (1999) Dynamics of disease resistance polymorphism at the *Rpm1* locus of *Arabidopsis*. Nature 400, pp. 667 – 671.

Steuernagel B, Periyannan SK, Hernández-Pinzón I, Witek K, Rouse MN, Yu G, Hatta A, Ayliffe M, Bariana H, Jones JD, Lagudah ES, Wulff BB (2016) Rapid cloning of disease-resistance genes in plants using mutagenesis and sequence capture. Nature Biotechnology. doi: 10.1038/nbt.3543.

Stewart HE, Taylor K and Wastie RL (1983). Resistance to late blight in foliage (*Phytophthora infestans*) of potatoes assessed as true seedlings and as adult plants in the glasshouse. Potato Research, 26, 363

Tameling WI, Elzinga SD, Darmin PS, Vossen JH, Takken FL, Haring MA, and Cornelissen BJ (2002) The tomato *R* gene products *I-2* and *MI-1* are functional ATP binding proteins with ATPase activity. Plant Cell 14, pp. 2929 – 2939.

Tan MYI, Hutten RCB, Celis C, Park TH, Niks RE, Visser RGF and van Eck HJ (2008) The *RPi-mcd1* Locus from *Solanum microdontum* Involved in Resistance to *Phytophthora infestans*, Causing a Delay in Infection, Maps on Potato Chromosome 4 in a Cluster of NBS-LRR Genes. MPMI Vol 21, Number 7, pp. 909 – 918. doi:10.1094 / MPMI -21-7-0909

Tanksley SD, Ganal MW, Prince JP, de Vincente MC, Bonierbale MW, Broun P, Fulton TM, Giovannoni JJ, Grandillo S, Martin GB, Messeguer R, Miller JC, Miller L, Paterson AH, Pineda O, Roder MS, Wing RA, Wu W and Young ND (1992) High density molecular linkage maps of the tomato and potato genome. Genetics 132, pp. 1141 – 1160.

Tian M, Win J, Song J, van der Hoorn R, van der Knaap E, and Kamoun S (2007) A *Phytophthora infestans* cystatin-like protein targets a novel tomato papain-like apoplastic protease. Plant Physiology 143, pp. 364 – 377.

Tomato Genome Consortium (2012) The tomato genome sequence provides insights into fleshy fruit evolution. Nature 485, pp. 635 – 641.

Torto AT, Li S, Styer A, Huitema E, Testa A, Gow NAR, van West P, and Kamoun S (2003) EST Mining and functional expression assays identify extracellular effector proteins from the plant pathogen *Phytophthora*. Genome Research, 13, pp. 1675-1685. doi: 10.1101/gr.910003

Tyler BM, Tripathy S, Zhang X, Dehal P, Jiang RH, Aerts A, Arredondo FD, Baxter L, Bensasson D, Beynon JL, Chapman J, Damasceno CM, Dorrance AE, Dou D, Dickerman AW, Dubchak IL, Garbelotto M. Gijzen M, Gordon SG, Govers F, Grunwald NJ, Huang W, Ivors KL, Jones RW, Kamoun S, Krampis K, Lamour KH, Lee MK, McDonald WH, Medina M, Meijer HJ, Nordberg EK, Maclean DJ, Ospina-Giraldo MD, Morris PF, Phuntumart V, Putnam NH, Rash S, Rose, JK,

Sakihama Y, Salamov AA, Savidor A, Scheuring CF, Smith BM, Sobral BW, Terry A, Torto-Alalibo TA, Win J, Xu Z, Zhang H, Grigoriev IV, Rokhsar DS, and Boore JL (2006) *Phytophthora* genome sequences uncover evolutionary origins and mechanisms of pathogenesis. Science 313, pp. 1261 – 1266.

van der Biezen EA and Jones JDG (1998). The NB-ARC domain: A novel signalling motif shared by plant resistance gene products and regulators of cell death in animals. Current Biology 8, pp. 226 – 227.

van der Hoorn RAL and Kamoun S, 2008) From Guard to Decoy: a new model for perception of plant pathogen effectors. The Plant Cell 20, pp. 2009 – 2017.

van der Linden CG, Wouters DC, Mihalka V, Kochieva EZ, Smulders MJ and Vosman B (2004) Efficient targeting of plant disease resistance loci using NBS profiling. Theorical and Applied Genetics, Vol 109, Issue 2, pp. 384 – 393. doi: 10.1007/s00122-004-1642-8

van der Vossen EAG, Gros J, Sikkema A, Muskens M, Wouters D, Wolters P, Pereira A and Allefs S (2005) The Rpi-blb2 gene from Solanum bulbocastanum is an Mi-1 gene homolog conferring broad-spectrum late blight resistance in potato. The Plant Journal 44, pp. 208 – 222. doi:10.1111/j.1365-313X.2005.02527.x

van der Vossen EAG, Sikkema A, te Lintel Hekkert B, Gros J, Stevens P, Muskens M, Wouters D, Pereira A, Stiekema W and Allefs S (2003) An ancient R gene from the wild potato species Solanum bulbocastanum confers broad-spectrum resistance to Phytophthora infestans in cultivated potato and tomato. The Plant Journal 36, pp. 867 – 882. doi:10.1046/j.1365-313X.2003.01934.x

van Poppel PMJA, Guo J, van de Vondervoort PJI, Jung MWM, Birch PRJ, Whisson SC and Govers F (2008) The *Phytophthora infestans* Avirulence Gene *Avr4* Encodes an RXLR-dEER Effector. MPMI Vol 21, Number 11, pp. 1460 – 1470. doi:10.1094 / MPMI -21-11-1460

Van Weymers PSM, Baker K, Chen X, Harrower B, Cooke DEL, Gilroy EM, Birch PRJ, Thilliez GJA, Lees AK, Lynott JS, Armstrong MR, McKenzie G, Bryan GJ and Hein I (2016) Utilizing "Omic" Technologies to Identify and Prioritize Novel Sources of Resistance to the Oomycete Pathogen *Phytophthora infestans* in Potato Germplasm Collections. Frontiers in Plant Science, Vol 7, Article 672. doi: 10.3389/fpls.2016.00672

Verzaux E (2010) Resistance and susceptibility to late blight in *Solanum*: gene mapping cloning and stacking. PhD Thesis, Wageningen University, The Netherlands.

Vleeshouwers VGAA and Oliver RP (2014) Effectors as tools in disease resistance breeding against biotrophic, hemibiotrophic, and necrotrophic plant pathogens. MPMI Vol. 27, No. 3, 2014, pp. 196–206. http://dx.doi.org/10.1094/MPMI-10-13-0313-IA.

Vleeshouwers VGAA, Raffaele S, Vosse JH, Champouret N, Oliva R, Segretin ME, Rietman H, Cano LM, Lokossou A, Kessel G, Pel MA and Kamoun S (2011) Understanding and exploiting late blight resistance in the age of effectors. Annual Review of Phytopathology 49, pp. 507 – 531.

Vleeshouwers VGGA, Rietman H, Krenek P, Champouret N, Young C, Oh S-K, Wang M, Bouwmeester K, Vosman B, Visser RGF, Jacobsen E, Govers F, Kamoun S and Van der Vossen EAG (2008) Effector genomics accelerates discovery and functional profiling of potato disease resistance and Phytophthora infestans avirulence genes. PLoS ONE Vol 3(8): e2875. doi: 10.1371/journal.pone.0002875.

Vos P, Simons G, Jesse T, Wijbrandi J, Heinen L, Hogers R, Frijters A, Groenendijk J,Diergaarde P, Reijans M, Fierens-Onstenk J, de Both M, Peleman J, Liharska T, Hontelez J and Zabeau M (1998) The tomato *Mi-1* gene confers resistance to both root-knot nematodes and potato aphids. Nature Biotechnology 16, pp. 1365 – 1369. doi:10.1038/4350

Wang M, Allefs S, van den Berg RG, Vleeshouwers VG, van der Vossen EA and Vosman B (2008) Allele mining in *Solanum*: conserved homologuesof *Rpi-blb1* are identified in *Solanum stoloniferum*. Theor. Appl. Genet. 116, pp. 933–943. doi: 10.1007/s00122-008-0725-3

Whisson SC, Boevink PC, Moleleki L, Avrova AO, Morales JG, Gilroy EM, Armstrong MR, Grouffaud S, Van West P, Chapman S, Hein I, Toth IK, Pritchard L and Birch PRJ (2007) A translocation signal for delivery of oomycete effector proteins into host plant cells. Nature 450, pp. 115 – 118.

Whisson SC, van der Lee T, Bryan GJ, Waugh R, Govers F and Birch PR (2001) Physical mapping across an avirulence locus of Phytophthora infestans using a highly representative, large-insert bacterial artificial chromosome library. Molecular Genetics and Genomics 266, Issue 2, pp. 289 – 295.

Wiesel L, Newton AC, Elliott I, Booty D, Gilroy EM, Birch PRJ and Hein I (2014) Molecular effects of resistance elicitors from biological origin and their potential for crop protection. Frontiers in Plant Science Volume 5, Article 655. doi: 10.3389/fpls.2014.00655

Witek K, Jupe F, Witek AI, Baker D, Clark MD and Jones JDG (2016) Accelerated cloning of a potato late blight–resistance gene using RenSeq and SMRT sequencing. Nature Biotechnology. doi:10.1038/nbt.3540

Xin XF and He SY (2013) *Pseudomonas syringae pv.* tomato DC3000: a model pathogen for probing disease susceptibility and hormone signaling in plants. Annual Review of Phytopathology, Vol 51, pp. 473 – 498. doi: 10.1146/annurev-phyto-082712-102321

Yandell M and Ence D (2012) A beginner's guide to eukaryotic genome annotation. Nature Reviews Genetics, Vol 13, Issue 5, pp. 329 – 342. doi:10.1038/nrg3174

Zhang C, Liu L, Zheng Z, Sun Y, Zhou L, Yang Y, Cheng F, Zhang Z, Wang X, Huang S, Xie B, Du Y, Bai Y and Li J (2013) Fine mapping of the *Ph-3* gene conferring resistance to late blight (*Phytophthora infestans*) in tomato. Theoretical and Applied Genetics 126, Issue 10, pp. 2643 – 2653. doi: 10.1007/s00122-013-2162-1

Zhang C, Liu L, Wang X, Vossen J, Li G, Li Tao, Zheng Z, Gao J, Guo J, Guo Y, Visser RGF, Li J, Bai Y and Du Y (2014) The *Ph-3* gene from *Solanum pimpinellifolium* encodes CC-NBS-LRR protein conferring resistance to *Phytophthora infestans*. Theoretical and Applied Genetics, Online ISSN: 1432-2242. doi: 10.1007/s00122-014-2303-1

Zipfel C (2008) Pattern-recognition receptors in plant innate immunity. Current Opinion in Immunology 20, Issue 1, pp. 10 – 16.

Published Work

Van Weymers PSM, Baker K, Chen X, Harrower B, Cooke DEL, Gilroy EM, Birch PRJ, Thilliez GJA, Lees AK, Lynott JS, Armstrong MR, McKenzie G, Bryan GJ and Hein I (2016) Utilizing "Omic" Technologies to Identify and Prioritize Novel Sources of Resistance to the Oomycete Pathogen *Phytophthora infestans* in Potato Germplasm Collections. Frontiers in Plant Science, Vol 7, Article 672. doi: 10.3389/fpls.2016.00672

Chapman S and Stevens LJ Boevink PC, Engelhardt S, Alexander CJ, Harrower B, Champouret N, McGeachy K, Van Weymers PSM, Chen X, Birch PRJ and Hein I (2014) Detection of the Virulent Form of AVR3a from *Phytophthora infestans* following Artificial Evolution of Potato Resistance Gene *R3a*. PLoS ONE Vol. 9, Issue 10: e110158. doi:10.1371/journal.pone.0110158